SYSTEMIC AND LOCAL HUMORAL IMMUNE RESPONSE IN PERIODONTAL DISEASES

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To my dearest Laura,
with all my love

DECLARATION

This thesis is the original work of the author.

John Mooney

List of Abbreviations

α1-M: alpha-1-antitrypsin

α2-M: alpha-2-macroglobulin

AP: adult periodontitis

ELISA: enzyme-linked immunosorbent assay

EU: ELISA units

GCF: gingival crevicular fluid

(M)GI: (modified) gingival index

ISH: *in situ* hybridisation

(L)JP: (localized) juvenile periodontitis

LF: lactoferrin

MANOVA: multivariate analysis of variance

MHC: major histocompatibility complex

PBS: phoshate-buffered saline

PBSE: phosphate-buffered saline containing 1% EDTA

PCR: polymerase-chain reaction

PD: probing depth

RCE: relative coefficient of excretion

RPP: rapidly progressive periodontitis

TIMP: tissue inhibitor of metalloproteinases

TF: transferrin

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SUMMARY

The precise role of the humoral immune response in periodontal diseases is not known. Previous workers have investigated the relationship between systemic antibody levels and disease status in great detail. However, areas such as local antibody levels, antibody avidity, differentiation of specific disease states, treatment effects and oral implants have been investigated to a much lesser extent. Therefore, in this study, these and other aspects were investigated in order to further elucidate the effects of the humoral immune response on the initiation and progression of periodontal disease.

An early study investigated systemic antibody levels to Gram-positive organisms during the course of experimental gingivitis. No significant changes in antibody levels were detected. However, at this stage, the ELISA technology employed was not sensitive enough to permit estimation of gingival crevicular fluid (GCF) antibody levels. Levels of α2-macroglobulin (α2-M) and transferrin were assayed to monitor the progression of inflammation.

Because many studies of the humoral immune response in periodontal disease have concentrated on antibody titre and very little has been published on antibody avidity, or binding strength, three studies were conducted into the relationship between antibody avidity and disease progression, disease state and response to treatment

respectively.

The first study assayed antibody avidities to *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* in adult periodontitis (AP) patients on a longitudinal basis. IgM antibodies to *P. gingivalis* were found to be higher in patients who did not go on to experience further attachment loss than in those who did. IgG avidities to *P. gingivalis* were significantly higher in AP patients than in control subjects, confirming a previous finding.

The second of these studies investigated the relationship between antibody avidity and periodontal disease classification, i.e. AP or rapidly progressive periodontitis (RPP). It had previously been shown that antibody avidities were lower in RPP patients than in controls, in complete contrast to the earlier finding for AP patients. However, this study demonstrated that IgG and IgM avidities to *P. gingivalis* were lower in RPP patients than in AP, when directly compared.

The third of these studies investigated the effect of periodontal treatment on antibody avidity. The scope of previous studies in this area was increased to include IgG, IgM and IgA avidities to both *P. gingivalis* and *A. actinomycetemcomitans*. The avidity of IgG antibodies to *P. gingivalis* and IgG, IgM and IgA titres to *A. actinomycetemcomitans* increased after therapy. Overall, the data suggest that therapy induces the production of higher avidity antibodies.

Modification of the ELISA technology by utilising biotin-avidin amplification increased sensitivity allowing antibody levels in GCF to be quantified. Four studies were conducted. Two of these were cross-sectional studies investigating healthy, gingivitis and periodontitis sites in periodontitis patients. Two compared cross-sectionally matched oral implant and natural tooth sites in oral implant patients.

The first of these studies demonstrated a strong correlation between IgG antibody levels to *P. gingivalis* in serum and GCF, confirming earlier findings. IgG levels to *P. gingivalis* were found to be significantly different in sites with different disease status, i.e. sites with deeper pockets and more inflammation had lower antibody levels than sites with shallower pockets and less inflammation.

The second of these studies focused on a comparison of IgG levels to *P. gingivalis* around oral implants and natural teeth. This is a field in which no previous work has been conducted on the humoral immune response, and very little on the inflammatory response. Therefore, assays of the acute-phase proteins, α2-M and α1-antitrypsin (α1-AT), and the iron-binding proteins, TF and lactoferrin (LF), were also performed. This study indicated great similarity between oral implants and natural teeth. However, there was also a suggestion that implants and natural teeth may differ in their local plasma cell infiltrate.

Because of the provocative conclusion of the first

study, that antibody levels were paradoxically lower in deeper pockets and more inflamed sites, it was decided to conduct another cross-sectional study in periodontitis patients. This differed in that three sites were sampled in each patient - one healthy, one gingivitis and one periodontitis. Paired comparisons were performed by multivariate repeated measures analysis of variance (MANOVA). In addition, levels of the tissue metalloproteinase, stromelysin, and tissue inhibitor of metalloproteinases (TIMP) were assayed to provide an indication of local tissue degradation.

IgG levels against *P. gingivalis* alone showed a significant difference between health and gingivitis. Additionally, there was a definite trend towards specific IgG levels being lower in periodontitis sites than in gingivitis sites; bearing out the findings of the previous study and suggesting that specific antibody levels may provide a more sensitive indicator of local disease progression than other parameters, e.g. metalloproteinases or TIMP.

A further comparative study of matched implants and natural teeth was extended in its scope to include all three main immunoglobulin classes directed against both *P. gingivalis* and *A. actinomycetemcomitans*. The findings accorded generally with those of the previous implant study and show that there is a tendency towards IgG titres to *P. gingivalis* and *A. actinomycetemcomitans* being higher in natural teeth and IgA titres to *A. actinomycetemcomitans*

being higher in implants.

Finally, a pilot study was conducted in which the relative coefficient of excretion (RCE) ratio was applied to assess the extent of local immunoglobulin production. The findings showed definite tendencies towards increased local IgG and IgA production and decreased IgM production in periodontitis.

In conclusion, this study has demonstrated that levels and avidities of systemic antibodies directed against particular suspected periodontopathogens are influenced by periodontal disease classification, disease status and the effect of treatment. GCF IgG titres to *P. gingivalis* may allow discrimination between gingivitis and periodontitis sites. The comparative studies of gingival tissue around implants and natural teeth have suggested, despite remarkable similarities in humoral response, that there may be differences in the plasma cell infiltrate around these fixtures. All of these topics would benefit from further research, involving advanced technologies such as *in situ* hybridization (ISH) and polymerase chain reaction (PCR), to further elucidate the periodontal humoral response.

Chapter 1

Introduction

1.1. Summary of relevant aspects of contemporary immunology

1.1.1. Introduction

Immunology has advanced at a rapid pace in recent years and many of these developments have impacted significantly on research into chronic immune and inflammatory diseases such as periodontal disease. These developments have been synthesized in a number of recent textbooks (1-5) and only a summary of the relevant points is presented here.

1.1.2. Innate immunity

Immunity to infection falls into two main categories, innate or non-specific immunity and acquired or specific immunity.

Mechanisms of innate immunity operate regardless of any prior contact with the invading organism. The simplest and most basic of these mechanisms are physical barriers preventing access to the body. These include skin which is impermeable, when intact, to most micro-organisms. In addition, mucus is secreted by membranes lining the inner surfaces of the body and this not only prevents adherence of micro-organisms to epithelial cells but also traps them allowing them to be eliminated mechanically by e.g. movement of cilia within the lungs.

The washing action of fluids such as tears, saliva and

urine also help keep epithelial surfaces clear of invading organisms and many of the secreted body fluids contain bactericidal agents e.g. lysozyme in tears, saliva and nasal mucus, lactoferrin and lactoperoxidase in milk, and hydrochloric acid secreted by the stomach. A similar beneficial washing function might be attributed to gingival crevicular fluid (GCF) also. This will be discussed at a later stage.

The normal bacterial flora of the body can also act as an effective buffer against infection by both passive and active mechanisms. They can inhibit the growth of pathogenic organisms by competition for nutrients or production of inhibitors. Commensal organisms in the gut can produce colicins. These bactericidins bind to the negatively-charged surface of susceptible bacteria and insert a hydrophobic, helical molecule into the membrane. This molecule then becomes completely hydrophobic and forms a voltage-dependent channel in the membrane, destroying the cell's electrical energy potential and leading to cell death.

1.1.3. Innate Immunity - Molecular Factors

If this first cohort of defence strategies is circumvented, the two main remaining innate mechanisms are those dependent on molecular factors and those dependent on cells.

Lysozyme is probably the most ubiquitous and abundant

of secreted anti-bacterials, a muraminidase which can split the peptidoglycan cell wall of susceptible bacteria.

Endotoxins produced by micro-organisms during infection stimulate the production of the cytokines, interleukin-1 (IL-1) and IL-6. IL-1 is an endogenous pyrogen (a general increase in body temperature being another innate defence mechanism), and can also, along with IL-6, stimulate the liver and other organs to produce molecules such as α1-antitrypsin (α1-AT), α2-macroglobulin (α2-M), both potent inhibitors of bacterial proteases, and C-reactive protein (CRP); proteins produced in this way as a response to infection are known as acute-phase proteins.

CRP binds in a Ca-dependent way to micro-organisms which contain membrane phosphorylcholine. Once complexed, the CRP-bound particle activates complement through the classical pathway resulting in binding of complement C3b to the membrane. This process, called opsonization (literally "making ready for the table"), renders the cell susceptible to adherence of phagocytes. Both phagocytic cells and complement will be discussed later. Interestingly, molecules such as CRP appear to be highly conserved in evolutionary terms, since the horseshoe crab's haemolymph contains a very similar analogue, limulin.

Finally, interferons are a group of broad-spectrum anti-viral agents. They are so named because they were first recognized by the phenomenon of viral interference. This is the mechanism by which an animal infected with one virus is rendered capable of resisting superinfection by

another virus.

Cells produce interferon when infected by a virus and secrete it into the extracellular fluid. The interferon then binds to uninfected cells. This promotes derepression of two genes in the target cell, permitting the synthesis of two enzymes. One of these is a protein kinase which catalyses the phosphorylation of a ribosomal protein and an initiation factor necessary for protein synthesis, thereby preventing mRNA translation to a large extent. The other catalyses the formation of a short adenylic acid polymer which activates a latent endonuclease. The latter has the ability to degrade both viral and host mRNA. The overall effect of this mechanism is to create a "cordon sanitaire" of uninfected and uninfectable cells around the locus of infection, thus ensuring containment.

However, as this mechanism can also inhibit host cell division as effectively as viral replication, it could have implications also in immune surveillance of neoplastic cells. By the same token, interferons could conceivably have a role in the healing phase of periodontal disease by "damping down" the immune and inflammatory reactions to bacterial products once the challenge has been removed.

1.1.4. Innate Immunity - Cells

Interferons may also modulate the activity of natural killer (NK) cells, which brings us to a discussion of the cellular components of innate immunity.

NK cells are large granular lymphocytes which have membrane receptors thought to be capable of recognizing structures on high molecular weight glycoproteins expressed on the surface of virally infected cells. Via these receptors, the NK cell is brought close to the target cell and becomes activated leading to polarization of granules between nucleus and target, and extracellular release of their contents into the intercellular space. Among the proteins released is perforin or cytolysin which bears some structural homology to complement C9. It can bind to the target cell membrane and form a transmembrane pore effecting apoptosis (programmed cell death) via a Ca-dependent endonuclease.

Another cell which effects extracellular killing is the eosinophil, a polymorphonuclear leucocyte which can bind to large parasites, e.g. helminths, coated with C3b. Most of these parasites are resistant to C9. The eosinophil granules contain a cationic protein and a major basic protein, both capable of damaging the parasite membrane, and also a protein capable of forming a transmembrane pore similarly to C9 or perforin.

The two main cells involved in killing of micro-organisms by phagocytosis, a form of total encirclement by the cell followed by intracellular destruction and digestion, are macrophages and neutrophils, the so-called "professional phagocytes".

Promonocytes in the bone marrow differentiate into mature monocytes which disseminate through the bloodstream

to locate in a number of tissues as mature macrophages. These macrophage populations make up the mononuclear phagocyte system, which comprises alveolar macrophages in the lung, cells in the lining of spleen sinusoids and lymph node medullary sinuses, Kupffer cells in the liver, and also macrophages throughout the connective tissue and around the basement membrane of capillaries. They also exist as osteoclasts in bone, mesangial cells in the kidney glomerulus and microglia in the brain. Macrophages are long-lived cells which can phagocytose invading bacteria directly. However, their particular specialism is their ability to phagocytose host cells infected with bacteria, protozoa or viruses.

The polymorphonuclear neutrophil (PMN) is a polymorphonuclear cell similar to the eosinophil but considerably more abundant, constituting about 70% of the leucocytes in the peripheral blood. These are short-lived cells produced in vast numbers in the bone marrow and spending about 36 hours in the bloodstream upon maturation before migrating into the tissues. PMNs contain three types of granules. The primary azurophilic granules contain myeloperoxidase, a group of cationic proteins and also some lysozyme. The secondary, so-called "specific", granules contain lactoferrin and large amounts of lysozyme. Acid hydrolases are contained in the tertiary granules. Since PMNs contain large glycogen stores which can be metabolized by glycolysis, these cells are ideally suited to operate under anaerobic conditions; a factor of crucial

importance in periodontal disease.

However, these cells do not operate in isolation. For example, activation and emigration of PMNs into a site of infection, e.g. a periodontal pocket, involves interaction between PMNs, macrophages and the complement system.

1.1.5. Complement

Complement comprises a complex series of 20 plasma proteins which constitute a triggered enzyme system similar in mechanism to the blood clotting, fibrinolytic and kinin-forming systems. Like these,it operates as a cascade where the product of one reaction then acts as an enzyme in the next. The net effect of this is to produce a rapid and highly amplified response to an initial trigger stimulus.

1.1.6. Neutrophil Activation and Emigration

Since the classical pathway is not an example of innate immunity, alternative pathway activation will be dealt with here to elucidate neutrophil activation and emigration. Lipopolysaccharides (LPS) and carbohydrates produced by bacteria react with receptors on the membranes of macrophages, e.g. the mannose/fucose receptor and the complement receptor. The mannose/fucose receptor binds surface carbohydrates of the bacterium whereas the CR3 complement receptor binds bacteria which have been opsonized with C3b via the alternative pathway.

Macrophages so stimulated release IL-1 and tumour necrosis factor (TNF), and synthesize and release neutrophil chemotactic factor (NCF; also designated IL-8).

These factors diffuse into the local vasculature and stimulate vascular endothelial cells to express the endothelial leucocyte adhesion molecule, ELAM-1, and up-regulate the concentrations of the adhesion molecules, ICAM-1 and ICAM-2. PMNs in the adjacent circulation are then bound by these adhesion molecules via their own adhesin, LFA-1. Movement of the PMN through the vascular endothelium and the basement membrane is modulated by IL-1 and TNF, this action being independent of any existing chemotactic gradient. However, the neutrophil's further journey through the subendothelial extracellular matrix occurs by chemotaxis through a chemotactic gradient, a form of directed migration. Chemotaxins involved here include IL-8, C5a, bacterially-derived formyl peptides, e.g. formyl-methionyl-leucyl-phenylalanine, and the leukotriene, B4.

These chemotaxins also activate the PMNs which can then destroy opsonized bacteria (i.e. with bound antibody/complement or complement only) by a number of oxygen-dependent mechanisms involving lipid peroxidation and the peroxide-myeloperoxidase system. As previously mentioned, PMNs can also kill using oxygen-independent mechanisms, e.g. neutral serine proteases (proteinases, elastase, cathepsin G), lactoferrin, lysozyme and defensins. These latter mechanisms are probably of much

greater relative importance in the anaerobic micro-environment of the periodontal pocket.

1.1.7. Acquired Immunity - B-lymphocytes

The cells mainly involved in specific or acquired immunity are antigen-presenting cells and lymphocytes. The former include macrophages, dendritic cells in the follicles of the lymph nodes, Langerhan's cells of the skin and mucous membranes, and B-lymphocytes themselves. These cells present antigen to B-lymphocytes and T-helper lymphocytes. The activated T-helper cells then produce cytokines which stimulate B-cells to differentiate into antibody-producing plasma cells.

Early workers in the field of antibody production postulated that antibody was produced as a malleable, uniform molecule which could be moulded to a specific structure by using the antigen as a template. However, Ehrlich (6), even a century ago, anticipated the mechanism which is accepted today, that of clonal selection by antigen. B-cells differentiate in the bone-marrow and are programmed to produce only one antibody. They display this on their surface as a receptor (about 10^5 molecules) but produce no further antibody (i.e. they are quiescent). When these antibody receptors are bound by antigen a triggering signal causes the B-cell to develop into an antibody-producing plasma cell.

The second step in this process is that the activated

B-cell proliferates to produce a clone i.e. a large number of identical cells producing identical antibody. This is known as clonal expansion and allows a high level of serum antibody to be produced within a few days.

A third feature of this process is that clonal expansion not only produces antibody-producing plasma cells but also a proportion of quiescent cells expressing antibody on their surface. These memory cells provide a relatively large pool ready to produce a much more rapid and intense antibody response on further antigen challenge.

1.1.8. Acquired Immunity - T-lymphocytes

T-cells act by another mechanism to destroy invading viruses, mycobacteria and protozoa which attempt to evade host defences by proliferating inside host cells. T-cells differentiate within the thymus and will only recognize foreign antigen when it is on the surface of a host cell in association with cell surface markers of the major histocompatibility complex (MHC). These regulatory cell surface molecules will be discussed in depth later.

Killing of virally-infected cells can be achieved by two mechanisms, one involving NK cells and the other cytotoxic T-cells. Apart from their direct effect on virally-infected cells, as already discussed, NK cells can also kill these cells by the process of antibody-dependent cell-mediated cytotoxicity (ADCC). NK cells have a receptor for the Fc portion of antibody and can thus bind

to antibodies directed against virally-coded surface antigens. The ADCC mechanism works well *in vitro*, but its relevance *in vivo* is difficult to assess.

However, a subpopulation of cytotoxic T-cells produces a wide range of surface receptors similar to the surface antigen receptors on B-cells. Similarly, these T-cells are programmed to recognize only one antigen when it is found in association with a class I MHC receptor. The cytotoxic T-cell can thus bind to the infected cell and destroy it.

T-helper cells will bind to an antigen for which they are programmed when it is found in association with a class II MHC receptor on the surface of an infected macrophage. They then produce lymphokines, including γ-interferon and other macrophage activating factors, re-triggering the microbiocidal mechanisms of the macrophage which had been repressed by the intracellular pathogen. Cytotoxic T-cells are also capable of acting in this way by producing γ-interferon. These actions of T-lymphocytes are referred to as cell-mediated immunity. In a similar fashion to that which pertains for B-cells, T-cells are selected and activated by antigen and expanded by clonal proliferation to produce a large clone of activated T-cells and also a pool of quiescent memory cells.

1.1.9. Regulatory Cell Surface Receptors

As previously discussed, the interactions between immune cells are not only influenced and controlled by soluble

agents, e.g. cytokines, complement and immunoglobulins, but also by regulatory cell surface molecules. These fall into four categories on immune cells: 1) MHC receptors; 2) cell surface receptors; 3) cluster of differentiation (CD) antigens; and 4) adhesins.

MHC antigens are transmembrane heterodimers. MHC class I molecules consist of a 43kDa peptide heavy chain non-covalently linked to a smaller 11kDa peptide called β-microglobulin. Most of the heavy chain is organized into three globular domains which protrude from the cell surface. A hydrophobic domain anchors the molecule in the membrane and a short hydrophilic sequence carries the C-terminus into the cytoplasm. Class II MHC receptors are also transmembrane glycoproteins, consisting of an α-chain of 34kDa and a β-chain of 28kDa. Class I receptors are found on virtually all cells, whereas class II receptors are found in B-cells and macrophages, and can be induced by γ-interferon on capillary endothelial and epithelial cells. In addition, a number of other genes within the MHC chromosome 6 region are known as MHC class III. These include genes coding for complement factors C2, C4 and factor B, and also TNF-α and -β. The MHC system has evolved as a highly polymorphic system based on multiple alleles (i.e. alternative genes at each locus). These multiple allelic forms are produced by various means including recombination, homologous but unequal cross-over, point mutation and gene conversion. This latter mechanism involves transfer of short nucleotide sequences from

apparently non-functional genes, e.g Qa, into functional ones.

The importance of MHC receptors in immunological phenomena has already been discussed, but they also have influence in developmental and hormonal events.

The T-cell receptor (TCR) merits further discussion. Like the MHC receptor it is also a heterodimer, comprising two chains of 40-50kDa each. Unlike B-cell receptors which exist as membrane-bound immunoglobulin M monomer, the TCR is obviously not synthesized by immunoglobulin genes. Functional T-cells display the TCR on their surface non-covalently bound to CD3 (one of the cluster of differentiation antigens). The seven-peptide chain CD3 transduces the antigen recognition signal into the cell.

Although the α and β chains of the TCR are not related to immunoglobulin, they share the same function. Each chain folds into two domains, one having a relatively invariant structure, the other displaying great variability akin to that of the immunoglobulin Fab fragment.

The CD antigens were discovered by immunizing animals with human lymphocytes. The antibodies produced recognize particular lymphocyte sub-populations. For example, CD3 is present on all T-cells but not on B-cells. CD4 is expressed on T-helper cells and CD8 on cytotoxic T-cells. These latter CD antigens act as co-receptors for class II MHC-antigen complex and class I MHC-antigen complex respectively, and are part of the TCR complex. CD4 is also a receptor for the human immunodeficiency virus.

The adhesins fall into four main groups: 1) integrins; 2) adhesion molecules of the immunoglobulin supergene family; 3) lectin-like adhesion molecules, the selectins; and 4) the CD44, or Hermes group, which interact with ligands called addressins on vascular endothelium.

Important integrins include CR3 and CR4 which bind to complement C3b and LFA which binds to ICAM-1 and ICAM-2. ICAM-1 and ICAM-2 are both found on capillary endothelium, and ICAM-1 is also expressed on B-cells and T-cells, and is probably involved in the interaction between antigen-presenting cells and lymphocytes. ICAM-1 is also probably important in transendothelial migration of PMNs.

Selectins include endothelial/leucocyte adhesion molecule (ELAM). Binding of neutrophils to ELAM is thought to be a crucial first step in neutrophil emigration across the capillary endothelium.

The CD44 homing receptors expressed on lymphocytes appear to be important in the binding of these cells to the high endothelial venules (HEV) of peripheral lymph nodes. The ligands for the CD44 antigen found on lymphocytes have been called vascular addressins. It is thought that there are other homing receptors selective for other lymphoid tissues, e.g. Peyer's Patch HEV, and, therefore, these receptors may be crucial in communicating lymphocytes to specific sites.

1.1.10. Immunoglobulins

An antibody, or immunoglobulin, molecule is made up of four peptide chains, two identical heavy chains and two identical light chains linked by interchain disulphide bonds. Immunoglobulin G (IgG) can be split by proteolytic enzymes to produce fragments giving clues to their function. The Fab fragment is now recognized as the antigen-binding fragment and the Fc as having other functions, e.g. complement binding. The Fab fragment contains considerable variability whereas the Fc fragment is relatively constant, and analysis of amino-acid variability has identified three hypervariable sequences on the heavy chain and three on the light chain in the Fab fragment.

Five different types of heavy chain exist in humans and these define the immunoglobulin classes, IgG, IgA, IgM, IgD and IgE. These are termed the immunoglobulin classes. IgG is the principal immunoglobulin produced in the secondary immune response. IgG diffuses more easily than the other immunoglobulins into the extracellular spaces where it has the predominant role in neutralizing bacterial toxins and binding to micro-organisms to promote complement fixation followed by phagocytosis.

The unique biological function of different immunoglobulin classes is mediated by the Fc portion. In the case of IgG, monocytes and macrophages contain receptors for Fcγ (FcγRI). FcγRII is found on monocytes,

neutrophils, eosinophils, platelets and B-cells. IgG complexes can bind to platelets causing thrombosis. Stimulation of B-cell Fc receptor leads to downregulation of IgG production, a possible mechanism whereby IgG and IgG immune complexes exert a negative feedback effect on antibody production.

The Fcγ region of IgG is responsible for complement activation via the classical pathway, binding to C1q and causing its activation.

Another low-affinity Fc receptor, FcγRIII, which is found on NK cells, macrophages, PMNs and eosinophils, is thought to be responsible for mediating ADCC by NK cells and immune complex clearance by macrophages.

IgG possesses the unique property among immunoglobulin classes of being able to cross the placenta providing considerable protection to the newborn in the first weeks of life, further supplemented by IgG in milk. The transport processes required here involve translocation of IgG across the cell barrier by complexing with another Fcγ receptor. A recent study has shown that the FcγRn receptor in the gut cells of the newborn rat complexes with IgG at relatively low pH in the gut lumen. This complex dissociates at the higher pH of the basal layer once the IgG has been transported across the intestinal cell (7).

IgA is adapted to defend the exposed external and internal surfaces of the body against micro-organisms. It is found predominantly in sero-mucous secretions, e.g. lung

secretions, saliva and secretions of the gastrointestinal and genito-urinary tracts. In these fluids it is present as a dimer which is stabilized against proteolysis by combination with the secretory component, which is synthesized by local epithelial cells. Dimerization occurs intracellularly via a joining sequence (J-chain), to prevent association of monomers with differing specificity. Essentially, IgA antibodies coat micro-organisms and thereby inhibit their adherence to mucosal cells. Aggregated IgA can also bind PMNs and can activate the alternative complement pathway. Monomeric IgA is also found in relatively high levels in the circulation indicating an additional systemic role for IgA.

IgM exists as a pentamer joined by a J-chain. IgM antibodies, although of low affinity as measured against single determinants, can demonstrate relatively high avidity, i.e. overall binding strength with molecules containing multiple epitopes. This is because the multivalency of IgM confers a synergistic effect whereby the binding of the molecule to two epitopes is of many times greater strength than the sum of the two interactions. In fact, the avidity is determined by a multiplication of the two binding strengths. This bonus effect of multivalency can be thought of as a vast reduction in the probability of simultaneous dissociation of several interactions as opposed to a single interaction.

IgM appears in the primary immune response and is extremely efficient in agglutination and cytolysis of

invading cells. As such it is thought to play a vital role in control of bacteraemia.

Immunoglobulins are further grouped into subclasses. IgG, for example, exists as IgG1, IgG2, IgG3 and IgG4, the difference being in the heavy chains which exist as γ 1,2,3 and 4 respectively. These heavy chain differences give rise to differences in biological properties. IgG2, for example, is relatively inefficient in placental transfer, whereas IgG3 is a very avid binder of complement, and IgG4 is a poor fixer of complement. IgG1 and IgG3 are both strong binders of monocytes. Some of these differences between different IgG subclasses may have an impact on the course of periodontal disease, and this will be discussed later.

The hypervariable regions on the immunoglobulin molecules which form the antigen-binding site are themselves, by definition, unique antigens which can be recognized by antibodies raised against them. In this context, these regions are referred to as idiotypic determinants; these compare with the isotypic determinants which define the various class and subclass variants and allotypes which define genetic variants of various regions.

Jerne's network hypothesis (8) states that because lymphocytes can recognize a vast array of foreign antigens, they should also be able to recognize the idiotypes of other lymphocytes. It is thought that a network may be formed which relies on idiotype-anti-idiotype recognition between lymphocytes of different subsets. The response to

foreign antigen would then affect the equilibrium of this network and provoke an appropriate response.

1.1.11. Generation of Diversity of Antibodies

Since the immune system must be capable of producing millions of different antibodies, the dedication of a single gene to programme for each antibody is clearly not an option as the mechanism producing such diversity.

To illustrate how such diversity is generated from a limited gene pool, the genes coding for heavy chains will be discussed here. The first element is that there are several genes coding for these; a single cluster is formed by the subclass constant region genes, and there is also a group of twelve highly variable D segments inserted between the V segment coding for the hypervariable regions and the J segment coding for a joining sequence to the constant region.

The diversity latent in this pool is considerably amplified by various mechanisms. The first of these involves rearrangement of these genes as the precursor cell differentiates into a mature B-lymphocyte. For example, there are 500 different V segments, 15 different D segments and 4 different J segments coding for heavy chain hypervariable region. Possible VxDxJ combinations generate thirty thousand rearrangements.

A second mechanism involves splicing out of base triplets upon recombination of V, D and J genes resulting

in removal of one amino-acid from the protein sequence. Splicing out of single base pairs and doublets can also occur resulting in a frame shift which thereafter codes for a completely different protein sequence.

A third mechanism involves random association between heavy chains and light chains, both of which have hypervariable regions. Since possible light chain combinations are on the order of 10^3, total heavy/light chain combinations are on the order of 10^7-10^8.

Other mechanisms amplify this diversity yet further, e.g. V gene swapping and reading of D genes in three different reading frames.

A final mechanism which deserves specific attention is that of somatic mutation. The V region rate of somatic mutation as a result of single base substitutions has been assessed at 2-4%, with mutations being more prevalent in IgG and IgA than in IgM. It is thought that somatic mutation is more involved in class switching and affinity maturation in association with immunological memory generation than simply in the generation of greater antibody diversity.

These and other mechanisms which tune the humoral immune response to pathogens in terms of avidity, subclass etc. during the course of periodontal disease will be further discussed later.

1.2. Microbiology of Periodontal Disease

1.2.1. Historical Perspective

Although references to periodontal diseases can be traced back to Roman physicians, oral microbiology can be said to date back to van Leeuwenhoek in 1772 (9). Later W.D. Miller, whose work was reprinted in 1973 (10), proposed his acidogenic theory of dental caries and, in addition, proposed that "pyorrhoea alveolaris" is not caused by a specific bacterium occurring in every case (as in classical single-pathogen infections e.g. tuberculosis), but that various bacteria may be responsible.

As such this was one of the earliest expressions of what is now known as the non-specific plaque hypothesis. This postulates that dental plaque can be considered a homogeneous mass which causes periodontal disease once it has accreted to a point where it can overwhelm host defence mechanisms. Similarly, one of the first suggestions of the specific plaque hypothesis was that of Bass who suggested a specific micro-organism, "Endoameba buccalis" as the cause of periodontal disease, even going so far as to suggest a vaccine to this organism as a form of therapy (11).

1.2.2. Infectious Nature of Periodontal Disease

Since the periodontal diseases, comprising gingivitis and

periodontitis, are caused by bacterial plaque, they are, therefore infectious diseases as borne out by much evidence summarized by Socransky (12). Notably, the classic experimental gingivitis study of Loe *et al* (13) demonstrated a correlation between the build up of plaque and the development of clinically demonstrable gingivitis.

Secondly, periodontal treatment which reduces plaque mass and removes some key species altogether can be correlated with clinical improvement.

Thirdly, *in vivo* and *in vitro* studies of plaque micro-organisms provide further evidence of the infectious nature of periodontal disease, especially with regard to the specific plaque hypothesis. A number of case reports have demonstrated various infections caused by oral micro-organisms in extra-oral sites (14, 15). Oral implantation into animals has been shown to cause periodontal disease with similar histopathology to that seen in humans (16, 17). Mixed infections have also been shown to have synergistic effects (18).

In vitro studies of pathogenicity have investigated the ability of plaque bacteria to produce virulence factors. Since Gram-negative bacteria tend to predominate in periodontal pockets, high concentrations of lipopolysaccharide (LPS) endotoxin tend to build up. This causes a cytotoxic effect on host cells, promotes osteoclastic bone resorption and results in a localized Schwartzman reaction with tissue necrosis.

Additionally, toxic metabolic end products are

released and there are indirect effects such as activation of the alternative complement pathway (19).

Certain bacteria, notably Porphyromonas gingivalis, also produce enzymes capable of destroying host proteins (20). *P. gingivalis* and *Actinobacillus actinomycetem-comitans* both produce collagenase which is capable of degrading host collagen. *P. gingivalis* also produces enzymes capable of degrading host immunoglobulins (21) and this organism, along with *Prevotella* ssp., has been shown to be able to coat itself with Fab fragments of degraded IgA1 thus evading host defences (22). Another example of this sort of strategy is the bacterial capsule which can inhibit phagocytosis by polymorphonuclear leucocytes (PMNs) and macrophages.

By the production of enzymes such as collagenase, hyaluronidase, gelatinase, aminopeptidase, proteinase etc., plaque bacteria can increase the permeability of the epithelium lining the gingival sulcus, destroy connective tissue and thereby cause proliferation of the junctional epithelium apically along the root surfaces.

1.2.3. Specific Microorganisms

The infectious nature of periodontal diseases having been clearly established, a number of studies have implicated specific micro-organisms in the aetiology of these diseases (23-27). Classically, a pathogen has been defined by Koch's postulates (28). Briefly, these three requirements

demand that 1) the agent must be isolated from every case of the disease, 2) the agent must not be isolated from cases of other disease states or from non-pathogenic (healthy) states and 3) after isolation and repeated growth in pure culture the agent must induce disease in experimental animals. However, Koch was required to abandon the third requirement in 1884 when he failed to induce cholera in animals, and in 1890 the second criterion was relaxed when the possibility of the carrier state was recognized.

Recent workers in periodontal microbiology have modified and extended Koch's postulates (27). The relevant criteria can be discussed under five headings. First, the criterion of association requires that the suspected pathogen be detected more frequently and at higher levels in disease cases than in controls. This would mean that the organism should be present in higher levels in actively progressing sites than in non-progressing sites, healthy sites or sites showing improvement. It might also be expected that a system of longitudinal monitoring would show an increase in the pathogen prior to or concomitant with clinically assessed disease progression.

Secondly, the criterion of elimination is based on the premise that periodontal treatment should influence not only the clinical course of the disease but also the associated micro-flora. A corollary of this view is that failure to eliminate or at least reduce the level of the pathogen would eventually lead to further progression at

that site.

Thirdly, the animal pathogenicity criterion continues to be used in the elucidation of possible pathogens, despite concerns about the use of animal models. They have been shown to produce valuable supportive evidence concerning the roles of certain micro-organisms in periodontal disease (29-31).

Fourthly, the response of the host to potential pathogens has been applied in attempts to differentiate the importance of various species in different forms of periodontal disease. This will be discussed in greater detail at a later stage.

Finally, some suspected organisms have the capability to produce virulence factors and these may not only be important in disease initiation and progression, but may also be useful as indicators of the pathogenic potential of the organism (32).

1.2.4. *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans*

Two organisms that have been particularly implicated in periodontal disease using these criteria are *P. gingivalis* and *A. actinomycetemcomitans*. Both of these organisms have been found in elevated numbers in periodontitis sites. However, *A. actinomycetemcomitans* has been associated particularly with the early-onset forms of periodontitis, localized juvenile periodontitis (LJP) and rapidly

progressive periodontitis (RPP). It has also been detected in prospective studies of disease progression. Further evidence of association is that *P. gingivalis* has been shown to bind to epithelial cells (27).

Elimination of both organisms results in successful therapy and recurrent lesions were found to harbour these species. Both species have been shown to induce periodontal disease in animal studies (27). The host response in terms of specific antibody production, both local and systemic, has been shown to be elevated with both organisms in different types of periodontal disease, a high titre to *A. actinomycetemcomitans* being associated with LJP. Both organisms also produce a number of virulence factors including enzymes such as collagenase, numerous proteases in the case of *P. gingivalis* capable of destroying most host proteins, endotoxin, leucotoxin and epitheliotoxin in the case of *A. actinomycetemcomitans*, and also factors inducing bone resorption and inhibiting PMN function (27).

1.2.5. Other Suspected Microorganisms

A number of other micro-organisms have also been implicated in periodontal disease. However, of these only spirochaetes, *Prevotella intermedia* and *Fusobacterium nucleatum* have been suggested as possibly causative satisfying all of the extended postulates (27). Spirochaetes are found in "superficial layers" of plaque

near host tissues, as evidenced by electron microscopy, where they are actively motile, suggesting that they have a strong affinity for host tissue. Indeed, they have been shown to invade gingiva in acute ulcerative gingivitis (AUG) (33). However, since spirochaetes do not form colonies on agar media but instead form "spirochaetal haze" as they move through the agar when cultured by membrane-filter and agar-well techniques, and are also sensitive to mechanical forces during sample dispersion or dilution and particularly to atmospheric oxygen, the laboratory culture of spirochaetes has been fraught with problems.

F. nucleatum is a Gram-negative obligate anaerobe which is frequently isolated from subgingival dental plaque in patients with adult periodontitis. Indeed, high numbers are often detected in sites undergoing active periodontal destruction. However, high numbers of *F.nucleatum* can also be isolated in inactive sites and, therefore, the role of this organism in periodontitis is equivocal (27).

Prevotella intermedia, like *P. gingivalis*, is frequently isolated from periodontitis sites. A recent study has demonstrated *Prevotella intermedia* in 88% of periodontitis subjects and *P. gingivalis* in 94% of these subjects. However, *Prevotella intermedia* was also detected in 42% of gingivitis subjects (i.e. no probing attachment loss > 2mm over one year of observation), whereas *P. gingivalis* was not detected in any of these subjects (34). Therefore, the association of *Prevotella intermedia* with periodontitis as opposed to gingivitis is also equivocal.

Since *P. gingivalis* and *A. actinomycetemcomitans* appear to be most strongly and unambiguously associated with periodontitis (35-38), the immune responses to these two organisms in particular were chosen for intensive study.

1.2.6. Current Concepts of Aetiology

In 1992 Socransky and Haffajee summarized current concepts of the aetiology of destructive periodontal disease in their review (39). Pathogenic species have been discussed above, and host susceptibility will be dealt with in the next section. However, the effect of beneficial species in the disease process is less clear, but it would seem reasonable to assume that non-pathogenic species could have a role in preventing disease initiation and progression by displacing pathogenic species.

Socransky and Haffajee conclude that the presence of a pathogenic species is necessary but not sufficient for disease to occur. In order that disease results from this pathogen 1) it must be of a virulent clonal type; 2) it must possess the chromosomal and extra-chromosomal genetic factors to initiate disease; 3) the host must be susceptible to this pathogen; 4) the pathogen must be in numbers sufficient to exceed the threshold for that host; 5) it must be at the right site; 6) other bacterial species must foster, or at least not inhibit, the process and 7) the local environment must be one which is conducive to the

expression of the species' virulence properties.

It may seem that the use of such extended requirements is an attempt to draw up a list of postulates which accord with what is already known about suspected periodontopathogens. However, their value can only be judged in truly prospective studies. In addition, the demonstration that virulence factors can be transmitted between different strains and even between different species, as evidenced by the transfer of antibiotic resistance between *P. denticola* and *P. intermedia* (40), may have enormous implications. The importance of bacteria in periodontal aetiology may in future be ascribed to genes coding for specific virulence factors rather than to particular microorganisms.

Socransky and Haffajee (39) conclude that diagnostic tests for bacteria should probably not exist in isolation but in combination with other diagnostic tests of host susceptibility. It is to this latter aspect in terms of humoral immune response that this study is applied.

1.3. Epidemiology and pathogenesis of periodontal disease and host responses

1.3.1. Introduction

Periodontal disease is a general term which can be used to refer to all diseases which could have an effect on the periodontium. Since the periodontium comprises tissues of different origin, both mesenchymal and ectodermal, a large range of systemic conditions with periodontal consequences could be included under this heading (41). However, in the present context, this term will only be used to denote gingivitis and periodontitis, i.e. dental plaque-induced inflammatory processes of the periodontium.

1.3.2. Gingivitis

In gingivitis, pathological changes are confined to the superficial gingival tissue, i.e. the gingiva. Clinical manifestations include redness and swelling, and an increased tendency to bleeding on probing, all associated with increased vascularity. The increased vascularity also leads to an increase in local temperature and increased gingival crevicular fluid flow. Gingival crevicular fluid (GCF) is an inflammatory exudate or preinflammatory transudate present in the gingival crevice. Its components are derived mainly from: 1) microbial products; 2) interstitial fluid and locally-produced factors of host

origin; 3) plasma; and 4) tissue degradation/turnover products (42). Production of GCF due to an inflammatory increase in the vascular permeability of the subendothelial vasculature was shown in early studies (43, 44).

1.3.3. Periodontitis

Periodontitis, however, also effects the deeper structures of the periodontium, i.e. cementum, periodontal ligament and bone attachment. This results in loss of periodontal support, bone loss as evidenced by radiographs and the development of the periodontal pocket. At least four different forms of periodontitis have been defined (45-47), and, as will be discussed later, it is thought that at least some of these have a basis in differences in host responsiveness.

Early studies in this field (48-50) contributed to the consensus that: 1) periodontitis is a virtually universal phenomenon affecting a very high percentage of the population; 2) the disease begins as gingivitis at an early age and if left untreated will develop into periodontitis; and 3) most of the perceived variance in periodontal disease is associated with age and deficient oral hygiene.

1.3.4. Recent Concepts in Epidemiology

However, recent studies have challenged these concepts and a different overall picture of the prevalence and

progression of destructive periodontal disease has emerged.

Recent cross-sectional studies have demonstrated that, although a periodontium of reduced height becomes the norm with increasing age, relatively few subjects in each age group suffer from advanced periodontal destruction. These subjects account for most of the sites which are severely periodontally involved (51-53). Papapanou *et al* reported that 75% of the total sites with previous attachment loss of at least 6mm occurred in 23% of the individuals examined (52).

Loe *et al* (54) reported similar findings in a 15-year-long study of the natural history of periodontal disease in Sri Lanka. These investigations identified a group of subjects, comprising 8% of the total sample, who showed rapid progression of periodontal disease, as defined by tooth mortality rates and interproximal attachment levels. In the same study, however, 11% of the subjects did not show progression of periodontal disease beyond gingivitis.

In addition, longitudinal studies have shown that relatively few sites undergo extensive periodontal destruction within a given observation period (55-57). Lindhe *et al* (56) reported that during a six year follow-up of 64 Swedish subjects, showing signs of previous periodontal destruction but not receiving active periodontal treatment, only 3.9% and 11.6% of their sites demonstrated attachment loss of greater than 2mm from baseline at 3 and 6 years respectively. Similar percentages (3.2%) of sites exhibiting periodontal

attachment loss were reported in the same study for a group of American subjects who were followed over a one year period. Moreover, it seems that relatively few individuals account for the sites which show active periodontal destruction over an observation period. Thus, Lindhe *et al* (57) in a recent study reported that 70% of the sites that deteriorated by 3mm or more during a two year monitoring period occurred in only 12% of the 265 Japanese subjects that were followed longitudinally.

1.3.5. Subject and Site Relatedness

Therefore, the current view is that periodontal disease is subject related, with a small subset of individuals within a given population suffering from advanced periodontal destruction, and with relatively few subjects and sites undergoing active periodontal destruction within a given period. In addition, in the absence of treatment, the mere presence of inflammatory periodontal lesions does not necessitate their progression (58). Thus, it has become apparent that any investigation of periodontal disease must take account of subject-specific and site-specific factors, and this will become clearer in the later discussion of subject and site susceptibility.

1.3.6. The Classical Model

Page and Schroeder (59) proposed five distinct states in the pathogenesis of periodontitis, ranging from health to advanced disease. The stages within this classical picture are health, the initial lesion, the early lesion, the established lesion and the advanced lesion. The initial PMN infiltrate was postulated to be rapidly replaced within 48 hours by a lymphocytic lesion. This lesion could then stabilise as the early lesion unless further challenged by plaque. With further plaque challenge, however, the cellular infiltrate was then thought to transform into a plasma cell dominated lesion. At this stage tissue destruction including bone resorption was thought to occur.

However, three factors have brought Page and Schroeder's model into serious question: 1)the inability to test the validity of the model with true longitudinal studies; 2)recent changes in ideas on periodontal disease activity; and 3) the fact that not all postulated stages have been observed histologically (60-64).

1.3.7. Three Models of Pathogenesis

Three models have been described to explain destruction of the periodontal supporting tissues: the continuous paradigm, the random burst theory and the asynchronous multiple burst hypothesis. The continuous paradigm postulates slow, constant and progressive destruction, and

is supported by cross-sectional studies (65, 66), and longitudinal monitoring of sites not responsive to treatment (67). However, as the study of Loe *et al* (66) shows, pooling data from groups, individuals and sites in cross-sectional studies can give the impression that destruction was slow and continuous.

The random burst theory proposes short periods of destruction punctuated by periods of resolution occurring randomly in time and at random sites within the subject (68). By contrast, the asynchronous multiple burst hypothesis proposes that destruction occurs within a defined time frame and then resolution or remission follows. This hypothesis suggests that many sites would show bursts of activity over a limited period of time and then these sites would become inactive indefinitely. None of these proposed mechanisms can be established or refuted by presently available data (69). However, a matter of some concern must be that methods of assessing parameters, such as attachment loss, generally yield such poor resolution. This means that such parameters will, almost by definition, lend themselves to the detection of "bursts" during longitudinal trials.

1.3.8. Stages in Pathogenesis

The Page and Schroeder model will be used as a framework for the following discussion of each stage in the

pathogenesis of periodontal disease, but conflicting views will also be presented where appropriate.

Under experimental conditions, clinically "healthy" gingiva can be established. The tissues are characterized by gingival index (GI) scores of 0 and a mere trace of gingival exudate (70, 71), but this is not identical with histologically "normal" gingiva (72). This normal condition only exists in gingiva adjacent to plaque-free teeth and is, therefore, rarely encountered in humans. It has been described in animals whose gingivae have been kept meticulously clean (73) and also in germ-free animals (74). It must be stressed that even biopsies of clinically normal human gingiva demonstrate infiltration of inflammatory cells. This infiltrate comprises 3-5% of the connective tissue volume contiguous with the junctional epithelium, and contains PMNs, monocytes, macrophages and lymphocytes, primarily T-cells with very few B-cells or plasma cells (59, 63, 75). This infiltrated area suffers from collagen depletion and its vascularity is increased. Fluid and plasma proteins leak from the microvasculature, percolating through the connective tissue and junctional epithelium into the gingival sulcus, giving rise to the GCF (76).

This stage constitutes one of the main problems in elucidating the pathogenesis of periodontitis. Investigators have been unable to make a clear distinction between normal and pathologically altered tissue. The crucial point at which disease commences has not been determined. Since definitive evidence on this point is

lacking so far, two hypotheses have been adduced. The first states that the features of the initial lesion merely reflect enhanced levels of the host defences occurring within the gingiva as part of normal surveillance procedures (59, 61, 77, 78). The second states that the "initial" stage may be a quiescent phase following destructive disease. As such it may constitute a later or indefinitely recurring stage in disease progression (79).

The acute, exudative, inflammatory response to plaque accumulation is known as the initial lesion (61, 78), according to the Page and Schroeder classification. The initial lesion usually manifests after 2-4 days, although vascular changes beneath the junctional epithelium can be seen within 24 hours of plaque accumulation; it is localized around the gingival sulcus. More blood is brought to the area by dilation of the arterioles, venules and capillaries of the dentogingival plexus. At the same time, intercellular spaces between capillary or venular endothelial cells appear because of the elevation of hydrostatic pressure. This causes increased permeability of the microvasculature resulting in leakage of fluids and proteins into the tissues. The result is the GCF flow. The fluid flow may act as a defence mechanism by flushing out bacterial products while, at the same time, delivering bactericidal host products, e.g. antibodies and complement.

Listgarten and Ellegaard (80) have shown that plasma cells predominate apically to the gingival sulcus in the initial lesion in animal models. However, in human models,

the initial lesion contains no plasma cells in the connective tissue area subjacent to the junctional epithelium (61, 81). It has been suggested that host defence mechanisms may not always have a beneficial role, and that they may also be destructive. Although neutrophils and macrophages are recruited to the area to defend the host against bacterial attack, their accumulation in the coronal part of the connective tissue and junctional epithelium probably accounts for much of the damage seen in this portion of the gingiva (82-85).

The initial lesion transforms into the early lesion within about one week of plaque accumulation (61, 86). The vessels of the coronal portion of the dentogingival plexus remain dilated (73, 76), and the additional plaque accumulation results in more pronounced infiltration of the dentogingival epithelium by PMNs and macrophages. The inflammatory cell infiltrate now occupies 10-15% of the connective tissue volume of the free gingiva (86) and contains T-cells and some B-cells (63). Lymphocytes predominate in the early lesion but few plasma cells have been demonstrated (61, 63, 64, 80).

The basal cells of the junctional/sulcus epithelium proliferate at this stage, and rete pegs have been demonstrated invading the coronal portion of the infiltrate (87, 88). The character of the cellular infiltrate and the nature of the pathological alterations observed have led to the concept that cellular hypersensitivity may be an important aspect of the early lesion. Wilde, Cooper and

Page (89) demonstrated that typical early lesions could be created in the gingival tissue of rats and monkeys sensitized to skin contact antigens followed by challenge at the gingival margin with the same antigen. They suggested a specific T-cell-mediated mechanism, since sensitization can only be transferred to unsensitized animals by means of lymphocytes, but not serum.

The duration of the early lesion has not been determined. Recently, a six-month experimental gingivitis study by Brecx *et al* (81) has demonstrated that more than six months of oral hygiene abstention is required before plasma cells dominate the lesion. Therefore, the early lesion may continue for a much longer period than previously thought.

The established lesion, as defined by Page and Schroeder, is one dominated by plasma cells. Many investigators have stated that only 3-4 weeks of plaque accumulation are required for the formation of a plasma cell dominated lesion (61, 90). However, this assumption has been challenged by Brecx *et al* (81).

The established lesion continues to exhibit the features of the preceding stages, but in a more marked degree (88, 91). Large numbers of plasma cells are seen (88, 92), which are primarily located in the coronal connective tissue and around vessels in more distal gingival connective tissue. Collagen loss continues in both apical and lateral directions as the inflammatory cell infiltrate expands, resulting in collagen-depleted areas

radiating deeper into the tissues (83).

Two types of established lesion appear to exist, some remaining stable and not progressing for months or years (93-96), while others become more active and convert to progressive destructive lesions. Controversy surrounds the nature of this conversion. Seymour, Powell and Davis (97) hypothesized that a change from T-cell to B-cell dominance presages the conversion from stability to activity involving aggressive destruction. However, Page (98) has disagreed with this view; a recent study has shown B-cell infiltrate mainly associated with stable, non-progressive lesions in childhood gingivitis (99).

The final stage in this process is known as the advanced lesion. The advanced lesion has characteristic features including alveolar bone loss and fibrosis of the gingiva with widespread manifestations of inflammatory and immunopathological tissue damage (100, 101). The lesion is no longer localized and the inflammatory cell infiltrate extends laterally and apically into the connective tissue. It is now generally accepted that plasma cells are the dominant cell type in the advanced lesion (100, 101).

1.3.9. Putative Immunological Mechanisms of Tissue Damage

Until fairly recently (102), discussions of the immunological mechanisms of tissue damage in periodontal diseases have referred to the four types of hypersensitivity reaction as classified by Coombs and Gell (103). However, the

characteristics of these do not accord well with the clinical and histopathological signs.

Type I (anaphylactic), type II (cytotoxic) and type III (immune complex) reactions are all antibody-mediated whereas type IV (delayed hypersensitivity) is cell-mediated. However, since B-cells and plasma cells predominate in the later stages of periodontitis, this cannot represent a pure type IV reaction.

Type I hypersensitivity reactions rely on IgE antibody bound to the surface of mast cells. Upon reaction with specific antigen, these cells release histamine and other inflammatory mediators. Mast cells have been demonstrated in the gingiva (104, 105), as has IgE (106), but type I reactions are probably not important elements in the immunopathogenesis of periodontal disease, as reflected in the scarcity of mast cells and IgE antibody in the periodontal tissues.

Type II reactions rely on complement-fixing IgG and IgM antibody. Damaged and lysing cells have been demonstrated in periodontitis (107, 108). This type of reaction is probably essentially protective. However, a recent study by Reinhardt *et al* (109) has shown a predominance of total IgG1 and IgG4 in active as opposed to stable periodontitis sites. Since IgG4 is relatively inefficient in fixing complement, two mechanisms suggest themselves here: 1) IgG is basically protective and a preponderance of IgG4 promotes disease progression by failure of antibody/complement mediated bacterial

destruction; and 2) a shift to IgG4 production locally may constitute an attempt to limit the potentially damaging effects of complement-mediated activity. Care should be taken in this interpretation, however, since immunoglobulin synthesized by gingival plasma cells has been shown to have specificity for non-oral bacteria (110) or non-bacterial antigens (111).

Type III reactions involve complement fixation by antibody-antigen complexes. However, since studies of complement in periodontal disease have demonstrated that these components are easily washed out, suggesting soluble proteins rather than fixed immune complexes (112), and attempts to extract insoluble immune complexes from periodontal tissue have not been successful (113), this type of reaction is unlikely to be important in periodontal destruction.

Another possible mechanism in the immunopathogenesis of periodontal disease is that of autoimmunity. Serum antibodies against type I collagen have been found in higher levels in periodontitis than in control subjects (114). A model has been presented whereby polyclonal activators initiate clonal expansion of autoreactive B-cells, followed by a specific response to stimulation by autoantigens produced by the initial response (115). However, there is a general dearth of evidence implicating autoimmune mechanisms in the pathogenesis of periodontal disease.

1.3.10. Role of Antibody-mediated Mechanisms

The role of antibody-mediated mechanisms will be discussed at greater length in a later section. At this stage, it seems reasonable to state that the role of antibody in periodontal disease is primarily protective, but that deficiencies or functional failure of local and/or systemic antibody may lead to subject and/or site susceptibility. The pathogenesis of periodontal destruction is probably determined to a large extent by the pro-inflammatory and tissue-degrading effects of cytokines released in response to bacterial products. Much evidence has been adduced demonstrating the ability of oral bacteria to directly stimulate cytokine production by macrophages (116-119). LPS from oral Gram-negative bacteria can markedly augment release of PGE_2 from cells activated by IFN-α (120), and polyclonal activators when present in optimal proportion to antigen can considerably amplify the secondary response to antigen (121). When one comes to consider the effects of these cytokines on metalloproteinase synthesis, prostaglandin release and bone resorption for example, the potential for pro-inflammatory and tissue-destructive events mediated by cytokines becomes obvious. The humoral immune response, insofar as it can bring about a reduction in bacterial antigen load, can be seen as acting against the promotion of tissue-destructive events.

1.3.11. Host Responses in Periodontal Diseases

To turn now specifically to host responses in periodontal diseases, it was demonstrated long ago that the gingiva adjacent to periodontal lesions is heavily infiltrated with mononuclear leucocytes. Models used in the earlier years of these studies suggested that the initial lesion is composed mainly of T-lymphocytes, with B-cells and plasma cells predominating at a later stage (63, 122, 123). However, this concept has recently been contested (124, 125). These studies found that both T-cells and B-cells may predominate in lesions, but that there was considerable variability in proportions of plasma cells. T4/T8 cell ratios are decreased in periodontal lesions compared with those seen in peripheral blood of periodontitis patients or in healthy gingiva (126, 127). A recent study has demonstrated that many of the CD4+ cells in periodontal lesions are in fact memory cells, and that CD8+ cells suppress and macrophages promote immunoglobulin production by plasma cells in the gingiva (128). By these mechanisms, regulation of local immune responses by gingival cells is made possible.

Responsiveness of leucocytes to various stimuli has also been extensively studied in periodontal disease. Polyclonal B-cell activation, which involves hyper-reactivity of B-cells to bacterial products as indicated by their blastogenesis leading to massive production of lymphokines and immunoglobulin, has been shown to be

increased in most periodontitis patients (115, 129). However, since this phenomenon cannot be demonstrated in about 30% of periodontitis patients, it may be that polyclonal B-cell activation offers another criterion by which these patients can be dichotomized into more susceptible and less susceptible groups. This concept will be dealt with further in the discussion of antibody avidity.

Elevated monocyte response to LPS has also been demonstrated (130, 131). These workers showed that periodontitis patients exhibit hyper-reactivity to LPS, presenting as increased monocyte PGE_2 and IL-1β production, and suggested that these patients may be susceptible to disease progression because of this state of hyper-reactivity.

Degradation of soft tissue in periodontal disease can be effected by both bacterial and host enzymes, including the matrix metalloproteinases. These include collagenase which can be produced by both fibroblasts and PMNs. The fibroblast type degrades collagen type III, whereas the PMN type degrades collagen type I. The stromelysins have multiple specificity and can also superactivate the collagenases. Gelatinase can degrade collagen types IV and V and also breaks down denatured collagen. Birkedal-Hansen *et al* have shown that at least three suspected periodontopathogens, i.e. *P. gingivalis*, *A. actinomycetem-comitans* and *Fusobacterium nucleatum*, produce factors that stimulate epithelial cells to degrade collagen fibrils

(132). The most likely mediator, in the case of *P. gingivalis*, is a thiol-dependent proteinase which activates mammalian procollagenase. In addition, the cytokines, IL-1β, TGF-α and EGF, can stimulate keratinocytes to produce type I and type IV/V specific collagenase (133). The potent ability of certain bacteria to precipitate tissue degradation, not only indirectly via cytokines, but also directly, further emphasises the crucial role of the host response in eliminating or limiting these pathogens.

From the above discussion it is clear that there is a relationship between immune function and the pathogenesis of periodontal disease. It has also been shown that different forms of periodontal disease show differences in their immunopathogenesis.

1.4. Specific Humoral Immune Response in Periodontal Disease

1.4.1. Introduction

When we come to consider the specific humoral immune response in periodontal disease, i.e. antibodies directed against particular oral microorganisms, there are a number of issues which must be addressed. First, the organisms to which a response is being detected must be considered. At this point microbial aetiology and pathogenesis must be taken into account since, as has previously been discussed, organisms may provoke an immune response but not fulfil other aspects of the extended postulates (39). In addition, antibodies may be detected to non-oral bacteria and non-bacterial antigens (110, 111). Some investigators have used a battery of microorganisms, including many of doubtful relevance, and this has led to an apparent lack of focus. In this study *P. gingivalis* and *A. actinomycetemcomitans* were chosen for particular attention because of their strong association with periodontal disease using all of the criteria previously mentioned.

Secondly, there has been controversy over whether whole bacterial cells should be used in assays of specific antibody, or particular antigens isolated from these cells (134). However, in this study, it was decided to employ whole cells because of the importance attached to another aspect of the antibody response, that of antibody avidity.

This can be seen as a global factor made up of the strength of interactions between numerous bacterial antigens and numerous host antibodies produced in response to these. Therefore, the overall response to a particular bacterium may yield information that would be lost by strict concentration on specific antigens, which may, in fact, not be virulence factors.

Thirdly, and related to the last point, there is the matter of antibody function. Clearly, demonstrations of an association between host antibody response and periodontal disease may be only academic unless it can be related to the biological function of this antibody. These functions, which include the ability to opsonize bacteria and the ability to bind strongly to fimbriae, which may prevent bacterial colonization, may relate to antibody avidity.

Fourthly, we must consider whether local antibody levels in the GCF or systemic levels or both are of importance; and whether local levels are merely a reflection of serum levels, or whether significant antibody production by gingival plasma cells is taking place. This is important in the consideration of subject and site susceptibility to disease onset and progression.

Fifthly, assessments of the titre and avidity of patient's antibody to suspected periodontopathogens may be useful in the differential diagnosis and classification of periodontal diseases.

Sixthly, the assay of these antibodies in longitudinal

studies may provide information on the relationship between antibody titre and avidity and disease progression at both subject and site levels, which may prove to be a marker of disease activity.

Finally, there is evidence that the subclass of immunoglobulin produced has a bearing on aspects of its function such as complement fixation and opsonization.

1.4.2. Response to Gram-positive Organisms

Most studies concerned with the humoral immune response to oral microorganisms have tended to concentrate on Gram-negative organisms, but there have been some which have focused on Gram-positive organisms; however few of these have been in recent years.

An earlier study by Ebersole's group assayed IgG and IgM antibody directed against *Actinomyces naeslundii*, *Actinomyces viscosus*, *Eubacterium brachy*, *Streptococcus mutans* and *Streptococcus sanguis* in localized juvenile periodontitis (LJP), generalized juvenile periodontitis (GJP), adult periodontitis (AP), acute ulcerative gingivitis (AUG), and edentulous patients and normal subjects. For all of these bacteria and all of these disease states there was no difference from the normal subject population (135). Similarly, this group went on to examine antibody titres to some of these organisms in Papillon-Lefevre syndrome, a type of neutrophil dysfunction manifesting with severe periodontal disease (136). In this

study no significant response to these organisms was detected. This does not suggest an important role for such organisms in this disease aetiology, since it might be expected that if Gram-positive antigens were penetrating the gingival tissues they would provoke a humoral immune response.

Page and Ebersole's groups also collaborated in a study which assayed levels of antibody to *Actinomyces naeslundii* in a family with a high prevalence of juvenile periodontitis (JP) (137). Elevated IgG levels to this organism were not detected although both patients and controls showed high levels of *Actinomyces* species in the subgingival microflora.

Ebersole's group have concluded that relatively abundant constituents of the Gram-positive flora in subgingival plaque, e.g. *Actinomyces* species, *Streptococcus mutans* and *Streptococcus sanguis*, do not provoke a significant humoral response even in patients whose microflora contain a large proportion of these organisms (138). They have also shown that these bacteria do not contain antigens that cross-react with Gram-negative constituents of the plaque (135, 139).

Studies of these organisms by other groups have also failed to demonstrate increased titres in periodontal disease (140-142). These have generally shown wide variation, lack of discrimination between diseased and healthy groups, and, in one study (140), higher levels of IgG and IgA antibody to *Streptococcus sanguis* in healthy

controls than in periodontitis sera. The only exception seems to be a study in 1970 by Nisengard and Beutner (143) showing higher levels of IgG antibody to *Actinomyces* species with increasing periodontal inflammation. However, the antibody assays in this study were by immunofluorescence and, therefore, the facility for quantification was probably inferior to that achieved in later ELISA-based studies.

Generally, differences in the humoral immune response to Gram-positive bacteria do not appear to occur between periodontitis patients and healthy controls. However, a notable exception may be the study of Tew *et al* (144) which examined antibody levels to *Eubacterium* species, *Lactobacillus minutus* and *Peptostreptococcus micros*; this being based on their previous study (145) in which they showed these organisms to be predominant members of the cultivable subgingival microflora in a cohort of patients with severe periodontitis. Their findings were that IgG antibody to *Eubacterium brachy* and *Peptostreptococcus micros* was raised in those patients but not in JP patients or healthy controls. However, it must be borne in mind that this study employed radioimmunoassay (RIA), rather than ELISA, which is known to be more sensitive, and refers to dichotomous measures of seropositivity/seronegativity rather than quantitative differences.

Therefore, it seems fair to conclude that there are no abnormal changes in the humoral immune response to the Gram-positive flora in periodontal disease; but that

constituents of this flora, e.g. *Eubacterium* species, although probably not aetiologic, may assume a more predominant role during the change to a pathogenic flora. There may concomitantly be an antibody response which could be a useful marker of this process. Accordingly, a section of the present study concerns the changes in antibody levels to certain Gram-positive organisms during an experimental gingivitis trial.

1.4.3. Response to Gram-negative Organisms

Studies of the humoral immune response to Gram-negative organisms have tended to target organisms such as *P. gingivalis* and *A. actinomycetemcomitans* because of their marked association with periodontal disease (27). An earlier study of particular relevance is that of Mouton *et al* in which serum IgG, IgM and IgA antibody to *P. gingivalis* was assayed in various patient groups, including AP, LJP and GJP (or RPP), and also in healthy controls (146). This report established that detectable levels of antibody to *P. gingivalis* are found in a significant proportion of healthy adults and also that there is a positive correlation between antibody levels and age. 80% of the healthy individuals studied had IgG antibody to this organism, 55% had IgM and 34% had IgA. Moreover, IgG and IgM serum antibodies were detectable in children as young as six months, and children aged 6-12 demonstrated significantly higher antibody levels than in younger

children. The authors concluded that this organism probably has an aetiologic role in the disease process, and that the antibody response is essentially protective.

In addition, mean levels of IgG antibody were found to be five times higher in AP patients and eight times higher in RPP patients than in controls. Higher IgM levels were seen in JP patients and higher IgA levels in RPP. Therefore, this paper was important in establishing a number of points:-

1. Based upon the humoral immune response, *P. gingivalis* is probably aetiologic in periodontal disease.
2. This response is probably protective.
3. Diseased and healthy individuals can be distinguished in terms of their antibody response to this organism.
4. There are indications of differences in the response in different periodontal disease states.

These findings were confirmed and extended by this and other research groups. Ebersole *et al* (147) studied 85 AP patients, 67 JP patients, 62 RPP patients and 82 controls, and found that 95% of JP patients had elevated antibody levels to at least one organism, predominantly *A. actinomycetemcomitans*. 48% of AP patients, and 37% of RPP patients had elevated serum antibody levels to *P. gingivalis*, whereas 85% of control subjects showed no elevations in antibody to any of the organisms in their battery.

Naito *et al* (148) assayed IgG antibodies to *P. gingivalis* in 42 AP patients and 17 controls. They graded

the AP patients according to disease severity and found a positive correlation of antibody level and disease severity. Although antibodies to *A. actinomycetemcomitans* were also elevated in this study, this was not significant, and, therefore, this was perhaps an indication of the differing relevance of these two organisms in AP.

1.4.4. Response to *Porphyromonas gingivalis*

Two studies by Ebersole *et al* (139) and Tew *et al* (144) examined antibody responses to various members of the *Bacteroides* genus as it was then classified. These included *Bacteroides gingivalis*, now *Porphyromonas gingivalis*, and *Bacteroides intermedius*, now *Prevotella intermedia*. Although elevated responses to other *Bacteroides* species were detected in some periodontitis patients, especially to *Prevotella intermedia*, *P. gingivalis* was found to be the most consistent example of a *Bacteroides* species eliciting an antibody response during periodontal disease; these responses being most frequent and of greatest magnitude.

Moreover, an investigation by Gmur *et al* (149) reported that IgG antibody levels to *P. gingivalis* were highly correlated with the extent of periodontal destruction, whereas anti-*Prevotella intermedia* reactivities were not. Similarly, studies by other groups have confirmed that antibody levels to *P. gingivalis* are higher in AP patients than in controls (150, 151).

Although these studies have tended to concur that an increased antibody response to *P. gingivalis* is associated with periodontal disease and may serve as an indicator, the protective nature of this antibody has been questioned. For example, Tollefsen *et al* (152) reported that immunoglobulin levels to *P. gingivalis* were lower in immunosuppressed transplant patients than in periodontitis patients, although the immunosuppressed patients also had a lower frequency of bleeding on probing and lower mean pocket depth than the periodontitis patients. They concluded that a reduced immune reactivity to plaque bacterial antigens leads to a milder and less destructive form of periodontitis. However, since both patient groups were not shown to be comparable in terms of previous periodontal disease experience, and the effects of immunosuppression on inflammation and vascularity could not be ruled out, it is difficult to see how such a conclusion can be drawn.

Generally, reports in this area have agreed that there is a positive relationship between serum antibody to *P. gingivalis* and other suspected periodontopathogens and periodontitis. However, there have been a significant number of studies reporting no difference between patients and controls or even lower antibody levels in patients (140, 144, 153-157). Although some of these studies are not particularly recent, and, therefore, may be seen as less accurate in their antibody assays, others, such as that of Chen *et al* (157) present a more significant

counterpoint to this argument. These workers studied RPP in particular and found that some patients mounted a humoral immune response to *P. gingivalis*, whereas others did not. In addition, these antibodies were of low avidity, but periodontal therapy could induce the production of higher avidity, and presumably more biologically effective antibodies. Thus, Chen *et al*, in their discussion, crystallize many of the issues which will be dealt with in this study in relation to the humoral immune response to *P. gingivalis*, e.g. patient susceptibility, diagnostic categories, treatment effects, and the importance of antibody avidity.

1.4.5. Response to *Actinobacillus actinomycetemcomitans*

An early study of the antibody response to *A. actinomycetemcomitans* linked this response specifically to LJP by demonstrating the presence of these antibodies in these patients but not in normal subjects (158). Ebersole *et al* also showed an association between increased levels of, and increased frequency of, antibody to *A. actinomycetemcomitans* and LJP (159). These authors elaborated on this in a later study (160) in which they showed a significantly increased level of IgG antibody to *A. actinomycetemcomitans* serotype b in 90% of LJP patients, but only 40% of RPP and 25% of AP patients. Listgarten *et al* (161) had previously made similar findings by immunofluorescent techniques. Several other reports have shown a strong

association between antibody to *A. actinomycetemcomitans* (especially serotype b) and LJP (155, 162, 163).

Zambon, in his review of 1985, stated that *A. actinomycetemcomitans* was an important organism in the aetiology of JP because of its increased prevalence in LJP patients and their families, and increased antibody responses to this organism (15).

Eisenmann *et al* (164) reported similar results with a Central African population and suggested that susceptibility to JP may have a genetic component related to a gene pool originally derived from Africa. This hypothesis was tested by a study examining the effect of race and periodontal status on antibody to *A. actinomycetemcomitans* (165). This showed a relationship between high levels of antibody to *A. actinomycetemcomitans* Y4 and both diagnosis of JP and black race.

Other studies in other ethnic groups have confirmed that *A. actinomycetemcomitans* is frequently associated with, and probably an aetiologic agent of JP, and may also be implicated in certain cases of RPP (166, 167). In addition, an investigation of anti-*A. actinomycetemcomitans* antibodies in individuals with AP showed no correlation between antibody levels and disease (149).

1.4.6. Response to other Gram-negative Organisms

Antibody responses to other Gram-negative organisms have generally failed to demonstrate any consistent and

convincing association between these responses and the occurrence and extent of periodontal disease. For example, Ebersole *et al* could not demonstrate any association between antibody levels to the *Capnocytophaga* species, *gingivalis*, *ochracea* and *sputigena*, and particular disease classifications (168, 169). Levels of antibody to *Fusobacterium nucleatum* have been shown to decrease after the age of twelve years in children with insulin-dependent diabetes (170), in contrast to the previously reported positively age-related antibody levels to *P. gingivalis*. *Eikonella corrodens* has been shown to provoke an antibody response during mono-infection induced periodontitis in gnotobiotic rats (171, 172). However, such studies are of doubtful relevance to human periodontal disease, not only because of the animal model employed but also because human periodontal disease involves mixed plaques rather than single organisms. It may be that some organisms, although capable of provoking periodontal destruction under artificial conditions, are normally limited in their effects by other plaque flora.

1.4.7. Response to Spirochaetes

There have also been some studies of antibodies directed against spirochaetes. Generally, these have not shown significant differences in antibody responses to organisms such as *Treponema denticola* between patients with periodontal disease and healthy controls (144, 173-175). Three explanations have been advanced to account for these

findings:-

1. Spirochaetes lack antigenicity.
2. Immunosuppression by spirochaete products.
3. Spirochaetal colonization is limited to subgingival plaque. Organisms and their products do not pass into host tissues.

However, spirochaetes have been shown to be antigenic in animals, and variable responses in the above studies demonstrate their antigenicity in humans. Also, immunosuppressive agents produced by spirochaetes have been shown to be generally suppressive *in vitro*. Therefore, the most probable explanation is that spirochaetes are limited to the subgingival plaque. The implication that spirochaete products cannot invade host tissues, although whole spirochaetes have been shown to invade the gingiva to a limited extent in AUG (33), does not suggest an aetiologic role. Spirochaetes have, however, been shown to be particularly associated with AUG. Even in this condition, however, no significant difference was seen in antibody levels to Borrelia vincentii between patients and controls (176).

1.4.8. Response to Particular Antigens

Some studies have investigated the humoral antibody response to particular antigens of bacteria rather than whole bacterial cells. However, intensive research into

the fimbriae of Gram-positive organisms such as *Actinomyces viscosus* (177-181), on the premise that anti-fimbriae antibodies could block adherence and thus colonization, do not appear to have taken into account the extensive literature showing that no significant response to these organisms occurs in periodontal disease. Similarly, a study showing differences in the ability of antibodies of various inbred strains of mice to bind to fimbriae of *Actinomyces viscosus* (182) is of doubtful relevance for the same reason, and also because of the equivocal contribution of such animal models to our understanding of the aetiology and pathogenesis of periodontal disease in humans.

Increased levels of IgG antibody to LPS of *P. gingivalis* have been demonstrated in periodontitis patients (135, 183). Another study demonstrated increased antibody levels to trypsin-like protein produced by *P. gingivalis* in periodontitis patients compared with control subjects (184). However, the ability of this antibody to inhibit this enzyme has not been shown.

There have also been investigations of the antibody response directed against fimbriae of *P. gingivalis*. Mouse monoclonal IgG1 antibody developed against *P. gingivalis* strain 381 inhibited adherence of *P. gingivalis* to buccal epithelial cells (185). Further work has shown that 67% of AP patients had antibody reactive with fimbriae, and that antibody activity could be detected to the 43kDa fimbrillin monomer and also to dimers, trimers and oligomers (186).

Ebersole *et al* have demonstrated differences in the antibody responses to particular membrane antigens of *P. gingivalis* (187). However, it should be noted that these antigens have not been identified as virulence factors, and patients with strong responses to these antigens often tended to be those who had a strong response to whole cells. Although these studies will undoubtedly help to elucidate the immune response to *P. gingivalis*, their use in clinical studies can be criticised on three points:-

1. Until particular antigens are identified as virulence factors, the demonstration of antibodies against these may be of doubtful relevance.
2. The results of some of these studies, e.g. that of Watanabe *et al* (188), have suggested that antibody reactivity to particular groups of antigens may be no more useful than to whole cells.
3. Focusing too narrowly on particular antigens may result in other valuable information being lost.

A study of antibody directed against LPS of *A. actinomycetemcomitans* found no increase in AP or JP patients over controls (189). By contrast, an investigation of the ability of antibody to neutralise *A. actinomycetemcomitans* leucotoxin found that 95% of JP patients demonstrated these antibodies as compared with much lower frequencies in other patient groups and control subjects (190).

Other studies have also dealt with antibodies directed

against leucotoxin (191, 192). However, the association between these antibodies and JP can also be shown in studies of antibodies to whole *A. actinomycetemcomitans* cells (166, 167), and the relevance of leucotoxin as a virulence factor has not yet been demonstrated *in vivo*.

1.4.9. Antibody Avidity

Antibody avidity, that is a measure of the net binding strength between antibodies and antigens, has been extensively studied in a number of fields, both in relation to antibody titre and in isolation, in terms of disease susceptibility and progression. For example, Morikawa *et al* (193) found that the titre of IgA to soybean antigen in the breast milk of Indian mothers was significantly higher than in Japanese mothers, although the avidity was significantly lower. Udhayakumar *et al* (194) have shown that monoclonal antibodies of higher avidity have much greater effectiveness in activating B-cells and presumably enhancing an immune response. Interestingly, Charoenvit *et al* (195) found that a monoclonal antibody to *Plasmodium yoelii*, a malarial parasite, had lower avidity for the antigen than vaccine-induced polyclonal antibodies in mice.

Doi *et al* (196) suggested that low avidity may be a pathogenic characteristic of IgG circulating immune complexes in membraneous nephropathy. Significantly, Joynson *et al* (197) found that toxoplasmosis patients with

acute infection had low avidity IgG to this organism whereas those with chronic infection had high avidity. This may have application in investigations of the phasic destructive episodes of chronic periodontitis, with the possibility that acute exacerbations and quiescent periods could be differentiated.

The secondary immune response classically generates antibodies of higher avidity. Francus *et al* (198) have demonstrated that a possible mechanism may be carrier primed T-cells which selectively activate virgin B-cells, which are then committed to the production of high avidity antibodies. A study with particular relevance to the suggested autoimmune aspects of periodontal disease is that of Panoskaltsis and Sinclair (199), who showed that autoimmune mice have lower avidity anti-single strand DNA than non-autoimmune mice.

There have, however, been very few reports dealing with antibody avidity in relation to periodontal disease. Ebersole *et al* (200) studied the increase in avidity in the non-human primate, *Macaca fascicularis*, following immunization with tetanus toxoid, which they used as a prototype bacterial exotoxin. They found that IgG avidity increased from 0.9M to 1.72M following primary immunization, and 2.56M after secondary immunization. Lopatin *et al* (201) demonstrated that avidity of antibody rose to a similarly high level in rabbits post-immunization with *P. gingivalis*, but that human antibodies to this organism appear to be of generally low avidity. They also

discovered that IgG antibodies to *P. gingivalis* were of significantly higher avidity in periodontitis patients than in control subjects (0.96M compared with 0.71M). They suggested that the presence of low-avidity antibodies contributes to the pathology associated with periodontal disease.

Chen *et al* (157) demonstrated that IgG avidities to *P. gingivalis* were lower in RPP patients than in control subjects. However, after treatment, the avidities increased significantly to levels higher than in controls. They concluded that many RPP patients do not produce protective levels of biologically functional antibody as a result of natural infection, but that treatment may induce the production of such antibodies. Another recent study of titre and avidity of IgG antibodies to *P. gingivalis* in RPP patients by Whitney *et al* (202) also shows lower avidities in RPP patients than in controls. A very recent study by Sjostrom *et al* (203) showed that IgG antibodies in low-titre sera from control subjects were significantly more effective in opsonizing *A. actinomycetemcomitans* than IgG antibodies in low-titre sera from RPP patients. This study suggests a crucial link between antibody avidity and function. Further support for this is derived from two recent reports demonstrating that effective binding of IgG to virulent *P. gingivalis* has a crucial role in the opsonization and phagocytosis of this organism and also in complement activation (204, 205).

A recent study by Ebersole and Kornman (206)

demonstrated that *P. gingivalis* emerges as an organism in the subgingival plaque during the conversion from gingivitis to progressing periodontitis in a non-human primate model, and that this elicits a systemic antibody response specific for this organism. Similarly, a study by Dahlen and Slots (18) in rabbits showed that animals co-inoculated with *P. gingivalis* and *A. actinomycetem-comitans* showed significantly more severe disease than animals which were monoinfected. They concluded that the immune system acting through systemic antibodies and/or cellular mechanisms may modulate the pathogenic potential of infecting periodontal pathogens. It may be that antibody avidity has a crucial role in this modulation.

1.4.10. The Local Response

Most studies investigating the humoral immune response have concentrated on systemic antibody levels. Much less work has been done on the relationship between local antibody levels and local disease status. Baranowska *et al* (207) found no significant difference in the level of specific IgG to *P. gingivalis* in GCF between healthy and diseased sites within the same individual. Tew *et al* (208) found no obvious differences in the clinical parameters of probeable depth and attachment level between sites with elevated antibody to *P. gingivalis* and/or *A. actino-mycetemcomitans*, and those with normal or low levels, and concluded that elevated antibody in GCF may relate to

changes in disease activity that are not detectable by normal clinical assessments.

However, Suzuki *et al* (151) demonstrated that local production of IgG to *P. gingivalis* was markedly increased in AP as compared with RPP patients, suggesting that disease progression is influenced by local antibody production. Challacombe *et al* (209) showed that levels of IgG antibody to *P. gingivalis* were lower in crevicular fluid washings (CFW) of patients with high periodontal disease index (PDI) as compared with those with low PDI, although this was not found to be statistically significant. Opsonic activity against *P. gingivalis* was, however, found to be significantly depressed in high PDI as compared with low PDI patients.

Lamster *et al* (210) reported a significant correlation between total IgG in GCF and specific serum antibody to *Prevotella intermedia* but not *P. gingivalis*. They concluded that the development of a serum immunoglobulin response to suspected periodontopathogens is consistent with a protective host response. A corollary of this view is that a local deficiency of IgG to these pathogens may lead to local disease progression. Alternatively, Killian (21) has demonstrated that *P. gingivalis* can degrade human IgG and IgA, suggesting that low GCF levels of IgG may be caused by degradation by this organism, or that locally available antibodies are adsorbed by the greater mass of subgingival plaque present.

An earlier study by Schenck (211) showed an inverse

relationship between the number of deep pockets (>4mm) and serum antibody level to *P. gingivalis* LPS in chronic periodontitis patients. Similarly, Mouton *et al* (212) demonstrated a dichotomy in serological responses to *P. gingivalis* among chronic periodontitis patients, with one subgroup exhibiting high serum antibody levels and another having levels similar to those of healthy individuals.

1.4.11. The Host Response in Differentiation of Disease States

The humoral immune response has been suggested as a means of differentiating between distinct periodontal disease states. A strong local and systemic antibody response to *A. actinomycetemcomitans* has been adduced as an indicator of JP (213-215). An association between the most severe and extensive cases of RPP and relative lack of antibodies to *P. gingivalis* and/or *A. actinomycetemcomitans* has also been reported (155, 216). This, coupled with the previously quoted findings that humoral responsiveness in RPP in terms of antibody avidity is depressed compared with that found in AP patients and healthy individuals, suggests possible disease mechanisms. When localized and generalized early-onset periodontitis (EOP) cases (i.e. JP and RPP) are grouped together and the extent and severity of disease is related to age and plaque index, there is a positive correlation for the RPP group for both factors. This

contrasts with the JP group for which the correlation is significant for plaque only (217). This would be consistent with an interdiction of disease progression in JP by an effective immune response, while those with an ineffective response would go on to develop generalized disease (RPP). Previous hypotheses have connected JP with PMN abnormalities (218-220), but these findings have been challenged recently (221-223). Thus this area would appear to be one in which fruitful progress may be made in enhanced differential diagnosis by objective tests of the humoral immune response.

1.4.12. Longitudinal Monitoring

In the most definitive study to date of longitudinal monitoring of humoral immunity in patients with destructive periodontal disease, Ebersole's group (224) studied 51 subjects bi-monthly for a period of up to five years. These comprised four subject groups which they defined as AP, LJP, GJP and RPP. They found that, in general, most serum antibody levels to subgingival species remained relatively constant for periods of up to five years. However, major increases and decreases in antibody to specific species could be detected in certain subjects. They concluded that major changes occurring in serum antibody may reflect fluctuations in the nature of the infection. In addition, they stated that their data

suggest that serum antibody levels to certain species over certain thresholds indicate an increased likelihood of disease activity taking place at some site in the oral cavity, and that it would be desirable to measure antibody to that species at the local sites in order to detect areas of active disease.

They further note differences between diagnostic categories and observe that the pattern of antibody fluctuation detected does not indicate a primary response, and that original antigen exposure probably occurred a long time in the past. This accords with the view of Lopatin *et al* (201) thatimmunological tolerance may be induced by asymptomatic bacteraemia associated with often severe pregnancy gingivitis (225-227), resulting in *in utero* exposure, and leading to depletion of high-affinity antibody-producing clones, leaving only low-avidity capability (228).

1.4.13. Immunoglobulin Subclass

A small number of studies have focused on the subclass of IgG which is produced against suspected periodontopathogens. Reinhardt *et al* studied total IgG subclass levels without investigating the specificity of these antibodies (109). IgG1 and IgG4 levels were found to be higher in active as compared with stable periodontitis sites with similar clinical characteristics. A more recent study investigated total IgG subclass levels

in periodontitis patients and matched controls (229). This found that IgG2 levels were significantly higher in patients than in controls, and these data suggested that the predominant antibody response to periodontal pathogens is directed against carbohydrate or glycolipid antigens.

Whitney *et al* (202) investigated the titre, avidity and subclass distribution of serum IgG antibodies to *P. gingivalis*. They found the subclass order in both patients and controls to be IgG2>IgG3>IgG1>IgG4. They concluded that their findings were consistent with the hypothesis that both carbohydrate and protein antigens are important in the IgG response to *P. gingivalis*. However, the relative predominance of IgG2, a subclass which lacks strong complement fixation and opsonic properties, and the low avidity of patient anti-*P. gingivalis* antibodies suggested that the humoral response to infection may be ineffective in clearing this organism.

Lopatin and Blackburn (230) have recently reported that adult periodontitis patients produced IgG antibodies to streptokinase (SK) and tetanus toxoid (TT) with much higher avidity than to *P. gingivalis*. However, when the IgG subclasses were examined, the IgG1 antibodies to this organism were found to be of similarly high avidity to those against SK and TT. Since IgG antibodies to *P. gingivalis* tended to be of the IgG2 subclass, which were of significantly lower avidity, the net effect of this predominating subclass was to lower the avidity of the overall IgG response to this organism.

1.5. Aims of the study

The overall aim of this study was to relate the systemic and local humoral immune response to periodontal disease status and progression. Thus, there are essentially two elements in this study.

The first involves an investigation of the relationship between clinical events such as experimental gingivitis, loss of periodontal attachment and response to periodontal therapy, and the systemic response to periodontal pathogens. In addition, the ability of antibody avidity to discriminate different periodontal disease states is investigated.

The second focuses specifically on the antibody response in GCF and peri-implant sulcular fluid (PISF). The relationships between local disease state and antibody titres to periodontopathogens are investigated, and comparisons between natural teeth and implants are made. Additionally, an investigation of the relative coefficient of excretion (RCE) is conducted in order to establish whether or not this measure of local immunoglobulin production can be related to local disease state.

Other parameters are also measured locally, e.g. acute-phase proteins, iron-binding proteins, albumin, stromelysin (a matrix metalloproteinase) and TIMP-1 (an inhibitor of matrix metalloproteinases). These other data allow assessment of inflammation (acute-phase proteins), PMN infiltration (lactoferrin), serum exudation into

GCF/PISF (albumin), and activation/inhibition of tissue destruction (stromelysin/TIMP-1).

Chapter 2

Materials and Methods

2.1. Experimental gingivitis study

2.1.1. Subjects and clinical protocol

Six dental student volunteers (3 male, 3 female), with no evidence of periodontal disease and clear medical histories, were entered into the study. Their ages ranged between 20 and 21 years. All subjects had more than 26 teeth, a high standard of oral hygiene and healthy gingival tissues. For two months prior to the study the subjects were regularly examined and their oral hygiene monitored to achieve maximal gingival health.

After determining baseline values the volunteers were instructed to stop all oral hygiene procedures for 14 days, after which they were given a thorough professional prophylaxis, instructed to recommence their normal oral hygiene procedures and were rechecked at 1 and 2 weeks post study. Clinical parameters and GCF samples were taken at Baseline and then at intervals 4, 7, 11, 14, 21 and 28 days from the same two sites in each patient, viz lower right lateral incisor, disto-buccal aspect, and the lower right central incisor, disto-buccal aspect. Clinical indices were recorded using the plaque index of Silness and Löe (231) (PI) and the modified gingival index (MGI). One examiner was used throughout the experiment to record the clinical indices.

2.1.2. Clinical indices

The Plaque Index of Silness and Löe (PI) estimates plaque on the four tooth surfaces (interproximal, lingual and labial) with a scoring system from 0-3. The tooth's PI is assessed by adding the scores and dividing by four. By adding the scores of each tooth and dividing by the number of teeth examined, the PI of the individual is assessed. The Lobene index scores marginal and interproximal tissues separately on a scale of 0-4. Lobene *et al.* (232) modified the gingival index of Löe and Silness (233) to eliminate the bleeding-upon-probing component to permit a non-invasive evaluation of the early visible changes in severity and extent of gingivitis. This index is termed the Modified Gingival Index (MGI). The mean MGI for each individual was calculated in the same manner as described above for the PI.

2.1.3. Gingival crevicular fluid sampling and processing

Whatman grade 4 paper strips were used for the collection of GCF, and are described by the manufacturer as a thin pure cellulose paper with an average thickness of 0.21 mm. The paper was cut manually using a steel ruler and a scalpel, into 2x13 mm strips, as recommended by previous investigators (234). A line was drawn at each strip at the length of 8mm indicating the length of the paper to be inserted between the periotron jaws.

The individual crevicular site was gently air-dried and any supragingival plaque was removed. The area was carefully isolated with cotton rolls so that no saliva contamination of the samples occurred. The paper strip was inserted into the crevice until mild resistance was felt, and was left there for 30s. Care was taken in order to avoid mechanical injury of the tissues. After the collection of the GCF, the paper strip was transferred to the chairside-located periotron (Periotron 6000, Harco Electronics, Winnipeg, Canada) for the quantification of the volume collected. The jaws of the periotron were wiped with pure methanol between sequential readings. The strips were then placed in individual sterile bijoux and stored at -30°C until further processing. Subsequently, the strips were eluted into 1 ml of phosphate-buffered saline with 0.05% Tween 20 (PBST) containing 0.1% bovine serum albumin for 1 hour using a rotating mixer, the strips were then discarded and the eluate was aliquoted and stored at the -70°C, until used. The 200 μl aliquots were analyzed for the quantification of α2-macroglobulin and transferrin.

2.1.4. Calibration of the Periotron 6000

Prior to commencement of this study a calibration graph was constructed for the Periotron in order to transform the digital indications for each filter strip into volumes and also to assess the accuracy of the instrument. Known volumes of serum dilution 1:1 in PBS were delivered to

Whatman grade 4 paper strips with a Hamilton microsyringe in volumes ranging from 0.2 to 1µl, in 0.2µl increments. Each measurement was performed 6 times and the mean value for each volume was used in a regression analysis, performed to construct the calibration graph, which was used for the determination of the volumes of gingival crevicular fluid collected.

2.1.5. Anti-bacterial antibody ELISA

The method was based on that of Ebersole *et al* (159). Briefly, *Streptococcus sanguis II, Actinomyces naeslundii* and *Fusobacterium nucleatum* were harvested after twenty-four hours growth on blood agar, or 7 days growth in ABB in the case of *F. nucleatum*. After washing in phosphate-buffered saline, 1mM Na_2 EDTA, pH 7.4 (PBSE), fixation for 1 hour in 10% formal saline and further washing and resuspension in a carbonate-bicarbonate coating buffer pH 9.6, 96-well ELISA plates were coated with the fixed bacteria at an optimal OD_{600} determined for each species. Overnight coating at 4°C and washing with PBST was followed by incubation with normal human serum serially diluted (base 2) and test samples. Ten normal human serum dilutions were used from the range 2-15 to yield the linear section of the curve. Test sera were diluted 1/100-1/4000 depending on the dilution profile of normal serum. This incubation was for 90 minutes at 37°C. Further washing

with PBST preceded the final incubation with HRP-anti-human IgG in the case of IgG estimation. IgA and IgM plates were incubated with anti-human-IgA (sheep) and anti-human-IgM (sheep) respectively, washed and then incubated with HRP-anti-sheep/goat IgG. These incubations were at 37°C for 90 minutes. o-Phenylenediamine (oPD) was used as a substrate for 7-15 minutes at room temperature. The reaction was stopped with 1M H_2SO_4 and the plates read at 490nm. All determinations were performed in triplicate and appropriate negative controls were employed throughout. Results were converted to ELISA units (EU) using the method of Gmur *et al* (149). This involved relating the sample absorbance to the absorbance of the reference serum at the same dilution. This latter value was arbitrarily set at 1,000 EU.

2.1.6. α2-Macroglobulin ELISA

The method was the indirect competitive ELISA of Altschuh and van Regenmortel (235). Briefly, 96-well ELISA plates were coated with α2-M at 1μg/ml in carbonate-bicarbonate coating buffer pH 9.6. Incubation was overnight at 4°C. After washing, serial dilutions of α2-M or test samples were added. The test samples were prepared by eluting the GCF strips into 1ml of the incubation buffer used in the assay for 1 hour at room temperature. An equivalent volume of anti-α2-M(goat) at 1μg/ml was also added. Incubation was for 2 hours at 37°C. HRP-anti-sheep/goat IgG was added after washing. Incubation was for 90 minutes at 37°C. Incubation with oPD for 15 minutes at room temperature

after washing allowed visualisation. The reaction was stopped with 1M H_2SO_4 and the plate read at 490nm. A standard curve was constructed allowing sample concentrations to be determined. These could then be related to GCF concentrations by employing the GCF volumes derived from fluid quantitation readings.

2.1.7. Transferrin ELISA

This was similar to the α2-M assay except for the substitution of transferrin and anti-transferrin antibody (sheep).

2.1.8. ELISA buffers

The buffers employed in ELISA assays throughout this entire study were as follows except where specifically mentioned in later sections:-

1. Coating buffer: 1.59g Na_2CO_3, 2.93g $NaHCO_3$ in 800ml distilled H_2O, dissolve, pH to 9.6 at just under 1L, add 0.2g NaN_3, make up to 1L in volumetric flask. Store in sterilized bottles at 4C for 1 week.

2. Wash buffer (x10 conc.): 80g NaCl, 14.4g $Na_2HPO_4.2H_2O$, 2g KH_2PO_4, 2g KCl, 5g Tween 20. Dissolve in 800 ml of distilled H_2O, make up to 1L and store at RT diluting 1/10 immediately pre-use.

3. Incubation buffer: 1/10 of the quantities added to above (2) made up to 1L with the addition of 1g of BSA. This should be layered on the surface without mixing. Store at 4C for maximum of 1 week.

4. Substrate buffer: 0.35g Na_2HPO_4, 0.2375g citric acid. Dissolve, make up to 25ml in measuring cylinder and add 1 tablet (10mg) of oPD and 10µl hydrogen peroxide, mixing well, immediately before use.

2.1.9. *In vitro* analysis of ability of organisms to eliminate host proteins

Periodontopathogens have previously been reported to be able to digest human proteins (20, 21), and in order to elucidate this an experiment was undertaken in which bacteria were incubated anaerobically at 37C with human serum or pure protein solutions (at 1mg/ml) for up to six days. Residual protein was assayed by ELISA, and related to 100% values, i.e. serum and protein similarly treated but without the presence of bacteria.

2.1.10. Reagents

All antibodies were obtained from the Scottish Antibody Production Unit (SAPU) except for anti-α2-M which was purchased from Sigma. α2-M and transferrin were also purchased from Sigma. ELISA plates were purchased from Dynatech.

2.2.Study of antibody avidity related to attachment loss

2.2.1. Bacteria

P. gingivalis NCTC 11834 was grown under anaerobic conditions (85% N_2, 10% H_2, 5% CO_2) and *A. actinomycetemcomitans* in CO_2 at 37C on Columbia blood agar. *P. gingivalis* was harvested after 5 days and *A. actinomycetemcomitans* after 24 hours into PBSE, washed by centrifugation, and fixed for 1 hour in 10% formal saline. The cells were then washed twice in PBSE and once in 0.1M Na carbonate-bicarbonate buffer containing 0.02% NaN_3 at pH 9.6 (coating buffer). Fixed cells were stored in coating buffer at 4C until use.

2.2.2. Subjects

Serum was collected from a total of 31 subjects. These included 11 periodontally healthy individuals and 20 periodontitis patients on maintenance phase therapy. The periodontally healthy individuals did not demonstrate significant gingival inflammation or attachment loss in any site. In addition, pocketing was ≤ 3mm in all sites. A serum sample was obtained for each healthy control subject.

The periodontitis patients were on a maintenance phase of therapy and had been diagnosed in the past as suffering from advanced periodontal disease (probing depth > 6mm in

at least four sextants). These patients had received a full course of periodontal treatment in the Glasgow Dental Hospital, were on maintenance for at least 1 year and had not received any antibiotics for 3 months prior to the initiation of this study. The periodontitis patients were followed longitudinally, over a period of three months, and serum was obtained at baseline and 3 months of the observation period. A minimum of 6 and a maximum of 15 sites were monitored in these patients and duplicate attachment level measurements were taken at the baseline and three month appointment. Attachment level change was determined using a prefabricated soft acrylic stent, the Florida probe stent handpiece (Florida Probe Corporation, Florida, USA) (236), and the tolerance method (237). The Florida Probe is a constant force (20g) electronic probe with a resolution of 0.2mm. The standard deviations of the difference of duplicate measurements, for subject, site and population were calculated (237). The attachment loss (AL) patients were those who demonstrated significant attachment loss surpassing the subject, site and population thresholds in at least one site during the three month observation period. Significant attachment loss at these sites ranged between 0.9 and 3.8mm (mean 1.91mm).

2.2.3. ELISA

Specific antibody titres were measured by ELISA based on the method of Ebersole *et al* (159), using formalinized

whole cells at an absorbance (OD_{600}) which had previously been determined as optimum to coat microtitre plates. Immulon 1 plates (Dynatech) were employed because of their low protein-binding characteristics. After coating, the plates were treated with PBS containing 0.1% bovine serum albumin (BSA), 0.05% Tween 20 and 5% skimmed milk to remove background binding. Serum serially diluted in this buffer, minus skimmed milk, was then added and the plates were subsequently incubated with biotin-conjugated anti-human IgG, IgA or IgM (Sigma) and thereafter with Extravidin-peroxidase (Sigma). Reaction was visualized using o-phenylenediamine dihydrochloride substrate and stopped with 1M H_2SO_4. Samples were assayed in duplicate and results were calculated using a regression line and derived equation from serial dilutions of a reference serum. Results were expressed as ELISA units (EU) (149).

2.2.4. Dissociation Assay

The dissociation assay to determine antibody avidity was performed as follows:- After incubation with serum as described above, the wells were treated with increasing concentrations of ammonium thiocyanate (0.2-8.0M). The concentration of thiocyanate required to dissociate 50% of bound antibody was determined by linear regression analysis. This was termed the ID_{50} and provides a measure of relative avidity as previously reported (238, 239).

2.2.5. Statistical Analysis

Two-sample t-tests were used to determine differences in avidity between different clinical groups. Mann-Whitney tests were used in group comparisons of the non-normally distributed antibody titres. Student's paired t-tests were used to assess whether differences were significant between baseline and recall appointments and to compare relative avidities of antibodies to *P. gingivalis* and *A. actinomycetemcomitans* in various sera.

2.3.Comparison of antibody avidity in various forms of periodontal disease

2.3.1. Subjects

Serum was collected from twenty-four adult periodontitis patients (AP), twelve rapidly progressive periodontitis patients (RPP) and twelve healthy control subjects who were age and sex matched to the RPP patients. Samples were collected from all patients before the commencement of therapy.

2.3.2. Statistical Analysis

Mann-Whitney tests were used in group comparisons of avidity where medians were employed and also in group comparisons of the non-normally distributed antibody titres. Student's paired t-tests employing the null hypothesis i.e. that difference = 0 were used to assess whether differences were significant between RPP data and that of age/sex matched controls. Regression analysis was used to correlate avidities and titres of antibodies to *P. gingivalis* and *A. actinomycetemcomitans* in AP, RPP and control (C) groups.

2.3.3. Other Methodology

As for Section 2.2.

2.4. Investigation of treatment effect on systemic antibody

2.4.1. Subjects

Nineteen adult periodontitis patients were studied. Serum was collected before treatment was initiated and after the completion of oral hygiene-phase therapy.

2.4.2. Statistical Analysis

Student's paired t-tests employing the null hypothesis i.e. that difference=0 were used to assess whether differences were significant between baseline and post-treatment samples.

2.4.3. Other Methodology

As for Section 2.2.

2.5. Comparison of local antibody titres in adult periodontitis patients (sites with differing clinical indices)

2.5.1. Subjects

The subjects were twenty patients with moderate periodontitis who were on recall maintenance therapy and had previously undergone active periodontal treatment. All patients were over 18 years of age with no history of systemic disease or history of antibiotic therapy within the previous three months. Five non-adjacent periodontal sites, previously selected by radiograph as being the most severely affected (greatest loss of periodontal bone) were sampled. These sites varied in disease status as defined by probeable depth and modified gingival index.

2.5.2. Clinical sampling technique

Sites for analysis were chosen on the basis of radiographic evidence and the order of sampling and clinical assessment was as follows. The modified gingival index (non-invasive) was recorded followed by careful supragingival debridement. Gingival crevicular fluid was sampled next, and finally pocket depth was recorded.

2.5.3. Microbiological culture technique

Each plaque sample was disaggregated by vortex mixing for 10 seconds. Dilutions of 1:10, 1:100 and 1:1000 were made in 1ml volumes of sterile anaerobe blood broth (Gibco-Paisley, Scotland). The two latter dilutions were spiral plated (Don Whitley, Shipley, England) onto a Columbia agar plate supplemented with 7.5% defibrinated horse blood and 1% v/v vitamin K/haemin solution. Actinobacillus selective agar plates (240) were inoculated with 50µl of the neat and 1:10 dilutions. The blood agar plates were incubated at 37°C in an anaerobic incubator (Don Whitley, Shipley, England) and examined after 7 days incubation. The Actinobacillus agar plates were incubated in a CO_2 incubator at 37°C within an atmosphere of 5% CO_2 in air for 2-3 days.

Following incubation, the total viable count was determined using a spiral system counting grid (Don Whitley Scientific, Shipley, England) as were the number of black pigmented colonies and the number of suspected Actinobacillus actinomycetemcomitans colonies. Wherever possible the whole plate was counted but where this was impossible, a segment of the plate was used. In order to balance any irregularities in sample deposition a similar segment on another part of the plate was counted. The anaerobic blood agar plate was also examined under UV light (365 nm) for colonies which produced red fluorescence (241).

A selection of pigmented colonies from the Actinobacillus agar, were subcultured to Colombia blood agar plates and incubated aerobically at 37°C in 5% CO_2. All Gram negative bacilli which were capnophilic and catalase positive were inoculated to an API 20A system (API lab products) to confirm identification of *A. actinomycetemcomitans* (242, 243).

2.5.4. Statistical analysis

Correlations between serum and gingival crevicular fluid were assessed by Spearman rank order correlation analysis. Comparisons between groups of sites were performed using Mann-Whitney tests for non-parametric data. Antibody titres were generally expressed as EU/30 second sample in order to eliminate inaccuracies caused by calculations involving very low or high volumes of gingival crevicular fluid beyond the limits which can be accurately recorded by the Periotron (244). In some cases, where direct comparisons between serum and gingival crevicular fluid were made, antibody titres were expressed as EU/ml.

2.5.5. Other Methodology

As for Section 2.2.

2.6. Comparison of humoral immunity in GCF and PISF

2.6.1. Subjects, sites and clinical indices

Thirty one partially edentulous subjects (18 males, 13 females; age range: 23 to 84 years), who had no history of systemic conditions which could influence the course of periodontal disease and had not been on antibiotics for the previous two months, were selected to participate in this study. The participants had at least two osseointegrated implants (*ad modum* Brånemark), which had been in function for a minimum of one year (average: 3.5 years; range: 1 to 10 years). Peri-implant sulcular fluid (PISF) was sampled from one healthy and one inflamed non-adjacent peri-implant site. In addition, 17 of the 31 patients contributed with one healthy and one inflamed tooth site from which GCF was collected. Out of the remaining 14 subjects, 4 contributed with one healthy and 10 with one inflamed tooth site only. Hence, a total of 21 healthy and 27 inflamed tooth sites were sampled for intra-subject comparisons between implants and natural teeth.

The non-invasive modified gingival index (MGI) (232) was used to assess inflammation and categorise the mucosa/gingiva at implants or teeth as healthy or inflamed. Sites with an MGI score of 0 or 1 were allocated to the clinically healthy group and sites with a score of 2 to 4, to the inflamed group. Presence of microbial deposits was assessed with the use of the Plaque Index system (PI)

(231). Following GCF and PISF sampling, probing depth (PD) and bleeding on probing (BOP) was assessed at implants and teeth using a pressure-sensitive periodontal probe with Williams markings (0.25N; diameter: 0.45mm; Electronic Periodontal Probe, Vine Valley Research, Middlesex, NY, USA). Dichotomous scoring was used for BOP.

2.6.2. GCF and PISF sampling

Sterile Whatman grade 4 (Whatman Labsales Ltd., Maidstone, Kent) paper strips (2x13mm) were cut using a steel ruler and a scalpel and a line was drawn at 5mm indicating the length of the strip to be inserted between the Periotron jaws. The individual crevicular site was gently air-dried in an apico-coronal direction and any visible supragingival plaque was removed. The area was carefully isolated with cotton wool rolls and a saliva ejector, to avoid salivary contamination of the samples. The paper strip was introduced into the crevice or peri-implant sulcus until mild resistance was felt or to a maximum of 1mm in deeper pockets. The strip was left *in situ* for 30s and then transferred, for volume determination, to the chairside located Periotron 6000 (Harco Electronics, Winnipeg, Canada), which was calibrated at each session using known volumes of phosphate-buffered saline in a 1:1 dilution with serum. The strip was then stored in a labelled sterile 1ml microcentrifuge tube, and placed on ice until all sampling was completed. The strips were subsequently transported

to the laboratory and stored frozen at -70°C until further processing. Prior to assaying, samples were eluted into 1ml of phosphate buffered saline containing 0.05% Tween 20 for 1h at room temperature.

2.6.3. Quantitation of GCF and PISF constituents

α2-M, α1-AT, TF, LF, albumin (Alb) and IgG against *P. gingivalis* in GCF and PISF eluates were assessed in the same sample using enzyme linked immunosorbent assays (ELISA).

The five sandwich ELISAs for α2-M, α1-AT, TF, LF and Alb are based on the technique described by Hetherington *et al.* (245) and modified by Adonogianaki *et al.* (246). In brief, the 96-well polystyrene microplate (Immulon 4, Dynatech Laboratories, Billinghurst, Sussex) was coated with the first antibody, a goat antiserum specific to the antigen to be quantified (1:6000 dilution in carbonate/bicarbonate buffer for α2-M, TF, LF and Alb; 1:3000 for α1-AT). The eluate of the sample was then added and any antigen present was captured by the immobilized antibody. This was followed by incubation with the second specific antiserum, developed in rabbit at 1:4000 dilution in incubation buffer, containing either 0.1% bovine serum albumin for the α2-M, α1-AT, TF and LF ELISAs (IB) or 5% skimmed milk for alb (IBM). Finally, the horseradish

peroxidase (HRP) conjugated anti-rabbit IgG (goat) was added (1:4000 dilution in the respective incubation buffer, IB or IBM). Visualisation was achieved by incubation with the substrate and stopping the reaction with H_2SO_4. The plate was read at 490nm. Plates included serial two-fold dilutions of purified antigen for the construction of a standard curve. Only the central wells were used when running standards or samples (in triplicate). The peripheral wells were used for assaying the controls. Prior to assaying with these sandwich ELISAs, GCF sample eluates were diluted further either in IB (or IBM when assaying for albumin), in order to achieve an optimal final dilution for each assay. The optimal dilution ranges for the samples, the working range for the standard for the α2-M, α1-AT, TF and LF ELISAs as well as the recovery rate for each of the acute-phase proteins from paper strips have been given elsewhere (247). The working range for the albumin ELISA was 125-1.95 ng/ml. Results were expressed either as ng/30s sample or ng/μg albumin.

2.6.4. Other Methodology

The method for determining specific antibody levels was as given in section 2.2. Results were expressed as absolute amounts in ELISA units (149) per 30s sample (EU/30s) sample or as specific amounts in EU/μg Alb.

2.6.5. Reagents

Purified α2-M ,TF and Alb were obtained from Sigma (Sigma Chemical Company Ltd., Poole, Dorset) whereas LF and α1-AT were purchased from Calbiochem (Novabiochem Ltd., Nottingham). Goat and rabbit anti-α2-M and anti-Alb, goat anti-TF, biotin-anti-human IgG and extravidin-peroxidase were also obtained from Sigma. Goat anti-LF as well as rabbit anti-LF and anti-TF were obtained from Nordic Immunological Laboratories (Maidenhead, Berkshire) whereas goat and rabbit anti-α1-AT were purchased from Calbiochem. The horseradish peroxidase (HRP) conjugated anti-rabbit IgG (goat) was purchased from ICN Immunobiologicals (Lisle, IL, USA). Of the above antisera the rabbit antisera to α2-M, α1-AT, the goat anti-TF and the HRP conjugates were fractionated.

2.6.6. Statistical analysis

In order to control for inter-patient variability only within patient comparisons were made. GCF or PISF results were logarithmically transformed in order to satisfy their distributional requirements ($\log_{10}$ (1+x) where x= original data value). Four separate multivariate repeated measures analysis of variance (MANOVA) procedures were applied to the logarithmically transformed data to test for all proteins simultaneously for differences between: a) healthy and inflamed peri-implant mucosa (n=31 pairs), b) healthy

and inflamed gingiva (n=17 pairs), c) healthy gingiva and peri-implant mucosa (n=21 pairs) and finally d) between inflamed gingiva and peri-implant mucosa (n=27 pairs). When a significant difference was detected by the analysis of variance, univariate paired t-tests were applied to the log transformed data to identify the location and direction of the statistically significant differences.

2.7. Cross-sectional study of local antibody levels

2.7.1. Subjects

A further cross-sectional study was performed on thirty adult periodontitis patients. One healthy site, one gingivitis site, and one periodontitis site were sampled for GCF as detailed in section 2.5.

2.7.2. Assay for stromelysin

This was based on the method of Cooksley *et al* (248). Extensive modification was carried out in order to increase sensitivity for use with GCF. Briefly, Immulon 4 plates were coated with a monoclonal antibody against human stromelysin. After overnight incubation at 4C, these were washed, blocked and incubated with serial dilutions of recombinant human stromelysin and eluted GCF samples in an incubation buffer containing 0.1% protease-free BSA (Calbiochem), for one hour at 25C with constant mixing. The plates were then washed and incubated with rabbit-anti-human stromelysin in the same buffer for one hour at 25C with constant mixing. The plates were then washed and incubated with HRP-anti-rabbit IgG (Jackson) in the same buffer for one hour at 25C with constant mixing. Finally, the plates were developed with 3,3',5,5'-tetramethylbenzidine (TMB) (KPL) for 15 minutes at 25C with constant mixing and reaction was stopped with 2.5% sodium

fluoride. The plates were read at 630nm. Sensitivity of 25pg/ml was achieved.

2.7.3. Assay for TIMP

This was based on the method of Cooksley *et al* (248). Extensive modification was carried out in order to increase sensitivity for use with GCF. Briefly, Immulon 4 plates were coated with a monoclonal antibody against human TIMP-1. After overnight incubation at 4C, these were washed, blocked and incubated with serial dilutions of recombinant human TIMP-1 and eluted GCF samples in an incubation buffer containing 0.1% protease-free BSA (Calbiochem), for one hour at 25C with constant mixing. The plates were then washed and incubated with another monoclonal antibody against human TIMP-1 which had been conjugated to biotin in the same buffer for one hour at 25C with constant mixing. The plates were then washed and incubated with Extravidin-peroxidase (Sigma) in the same buffer for one hour at 25C with constant mixing. Finally, the plates were developed with 3,3',5,5'-tetramethylbenzidine (TMB) (KPL) for 15 minutes at 25C with constant mixing and reaction was stopped with 2.5% sodium fluoride. The plates were read at 630nm. Sensitivity of 25pg/ml was achieved.

2.7.4. Reagents

All antibodies and proteins for the above two assays,

except where otherwise mentioned, were kindly supplied by Dr AJP Docherty, Department of Immunochemistry, Celltech Ltd., Slough, U.K. Protease-free BSA was obtained from Calbiochem-Novabiochem, La Jolla, CA, USA. HRP-anti-rabbit IgG was obtained from Jackson Immunoresearch Laboratories, West Grove, PA, USA. TMB was obtained from KPL Laboratories, Gaithersburg, MD, USA.

2.7.5. Other Methodology

As given in Section 2.5.

2.8. Cross-sectional comparison of natural teeth and implants

2.8.1. Subjects

Twenty partly edentulous patients with osseointegrated implants were studied. All of these patients had previously suffered from moderate to severe periodontitis, which was treated before placement of implants. The following inclusion criteria operated:-

1. No bleeding on probing.
2. Probing depths less than 4mm.
3. Absence of visible plaque (PI=0).
4. Clinical and radiographic signs of osseointegration present at implant sites.
5. Implants placed at least six months previously.

Implant and tooth sites were selected and matched according to probing pocket depth and modified gingival index.

2.8.2. Other Methodology

As for Section 2.5.

2.8.3. Statistical Analysis

Paired t-tests were performed on the log transformed data

using the null-hypothesis (i.e. that there is no difference between implant and natural tooth).

2.9. Potential of RCE to distinquish healthy and periodontitis sites

2.9.1. Introduction

This was a small study of ten healthy subjects and five periodontitis subjects in which GCF was sampled from two sites in each subject. Total IgG, IgM, IgA and albumin was assayed in both GCF and serum and the RCE value as used by Giannopoulou *et al* (249) and Out *et al* (250) was calculated. The formula applied was as follows:-

$$RCE = \frac{\frac{Ig(GCF)}{Ig(serum)}}{\frac{albumin(GCF)}{albumin(serum)}}$$

This ratio gives a value directly proportional to the extent of local immunoglobulin production.

2.9.2. Total Immunoglobulin Assays

The three sandwich ELISAs for total IgG, IgM and IgA concentration in serum and GCF are based on the technique described by Hetherington *et al*. (245) and modified by Adonogianaki *et al* (246). Briefly, Immulon 4 plates were coated with anti-human IgG or IgM or IgA (sheep). Plates were then washed and blocked with incubation buffer (IB) containing 5% Marvel (IBM). The plates were then incubated

with serial dilutions (1/2) of normal serum of known concentration beginning at 1000ng Ig/ml, together with diluted test sera and GCF samples diluted as given below. There then followed an incubation with biotin-anti-human IgG or IgM or IgA. Finally, plates were incubated with Extravidin-peroxidase. Reaction was visualised using oPD substrate. Reaction was stopped with 1M H_2SO_4 and plates read at 490nm.

2.9.3. GCF sample dilutions

1. GCF strips were eluted into 500ul of IB without BSA for 1hr at RT on rotary mixer.
2. A 20ul aliquot was removed for albumin assay and 500ul of IB with 0.2% BSA was added (total volume 980ul).
3. The sample was mixed well and aliquoted in 120ul volumes. Aliquots stored at -30C.
4. The dilutions of eluted GCF samples for assay were as follows:-

Volume	IgG	IgM and IgA
v < 0.1ul	1/10	1/4
0.1 < v < 0.2	1/25	1/10
0.2 < v < 0.4	1/50	1/20
0.4 < v < 0.8	1/100	1/40
v > 0.8	1/200	1/80

2.9.4. Albumin Assay

This was performed as detailed in Section 2.6.

2.9.5. Data Analysis

The reliability of the RCE was assessed using parameters adapted by Lang *et al* (251). These were:-

1. Sensitivity = $\frac{\text{true positives}}{\text{true positives + false negatives}}$

2. Specificity = $\frac{\text{true negatives}}{\text{true negatives + false positives}}$

3. Accuracy = $\frac{\text{true positives + true negatives}}{\text{all results}}$

2.9.6. Other Methodology

As given in Section 2.2.

Chapter 3

Results

The results of the investigation into the humoral immune response will be presented as studies of the systemic antibody response and of the local antibody response.

3.1. Systemic Response

3.1.1. Experimental gingivitis study

A study was performed on six subjects undergoing a 14-day experimental gingivitis trial. The systemic antibody response to the three organisms most commonly isolated during this study (data not shown) is detailed in Table 1. No significant changes were observed during the 28-day monitoring period. Another element of this investigation was to establish whether or not GCF levels of the protease-inhibitor, α2-macroglobulin (α2-M) and the iron-binding protein, transferrin (TF) change with regard to clinical indices. These data are shown in Figure 1. α2-M levels peak after 4 days and then fall off, showing a similar pattern to that previously reported for interleukin 1 (IL-1) (252). In contrast, TF levels fall off constantly during the trial and do not begin to recover until day 28. The results of the *in vitro* analysis of the ability of organisms to eliminate these host proteins are shown in Table 2. These demonstrate the relative efficiency of *P.*

Table 1: Median antibody titres (EU) at baseline and day 28.

	IgG		IgM		IgA	
	Baseline	Day 28	Baseline	Day 28	Baseline	Day 28
Streptococcus sanguis	**520**	**521**	**341**	**388**	**586**	**644**
Fusobacterium nucleatum	**856**	**758**	**849**	**644**	**260**	**244**
Actinomyces naeslundii	**283**	**277**	**260**	**244**	**335**	**362**

Table 2: Effect of suspected periodontopathogens on elimination of transferrin and α-2-macroglobulin in serum and pure protein solution.

% protein remaining	Transferrin (serum)	Transferrin (protein)	α-2-Mac (serum)	α-2-Mac (protein)
Streptococcus sanguis	**88%**	**100%**	**100%**	**100%**
Actinomyces naeslundii	**51%**	**100%**	**100%**	**100%**
Fusobacterium nucleatum	**40%**	**100%**	**74%**	**100%**
Porphyromonas gingivalis	**2%**	**4%**	**25%**	**46%**

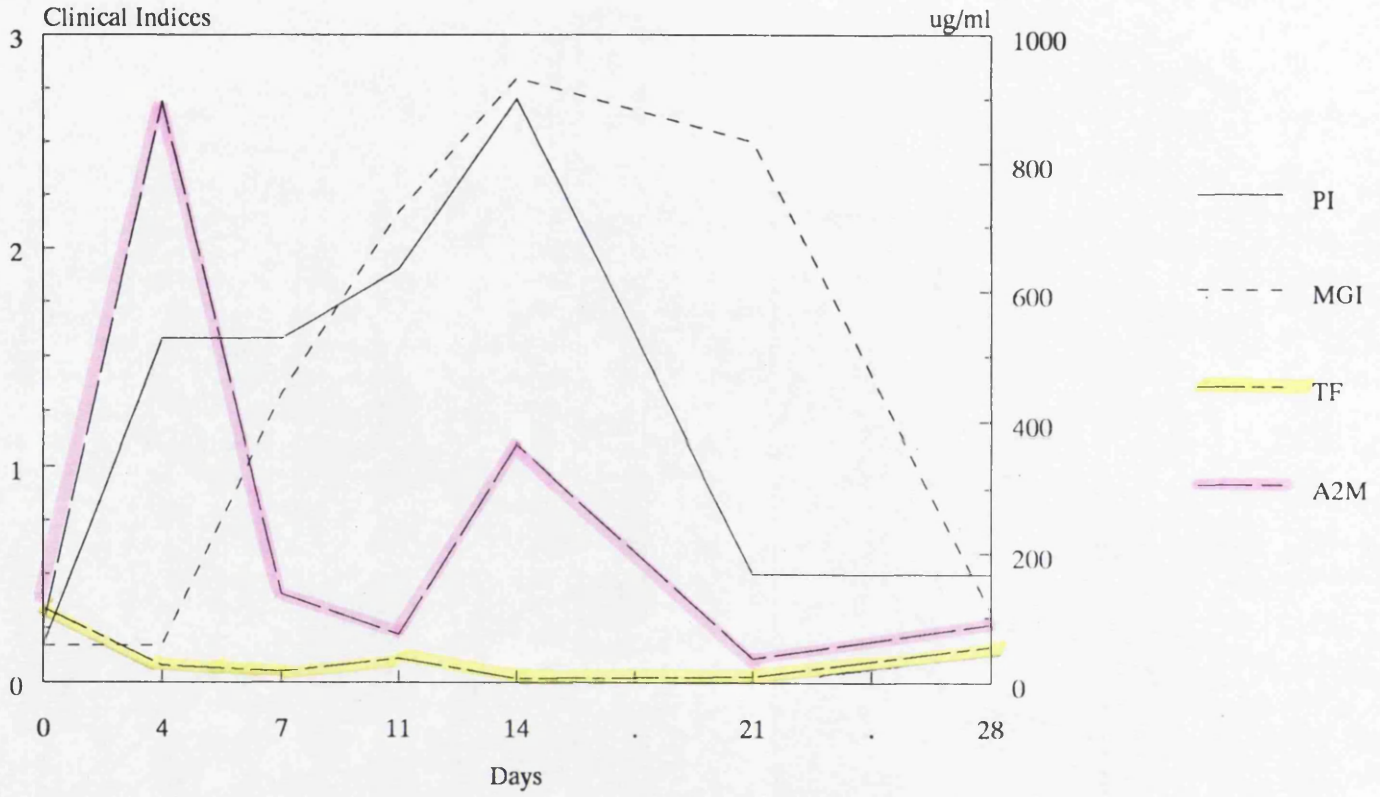

Figure 1: Clinical indices and GCF protein concentrations during experimental gingivitis study.

gingivalis, which may be attributed to degradation and/or adsorption.

3.1.2. Study of antibody avidity related to attachment loss

ELISA was used to determine both the avidity and titre of IgG, IgA and IgM antibodies to the Gram-negative anaerobe, *P. gingivalis*, in twenty periodontitis patients enroled in a longitudinal study of attachment loss and eleven non-periodontitis affected subjects. A cross-sectional analysis of the longitudinal patients at baseline and non-periodontally affected controls confirmed earlier findings that IgG and IgA antibody titres to this organism were higher in periodontitis patients than in individuals who were not periodontally affected. In this cross-sectional analysis, IgG antibody avidities were not found to be significantly higher in periodontitis patients than in control subjects ($p=0.065$). However, indications of the potential prognostic value of antibody avidity were demonstrated by the higher IgM avidities in patients who did not experience attachment loss during the three-month monitoring period than in those who did ($p=0.0005$).

The periodontally healthy (n=11) and maintenance periodontitis groups were compared in terms of titre and avidity of IgG, IgM and IgA class antibodies to *P. gingivalis*. The periodontitis group was also sub-divided into a sub-group of 14 patients who experienced attachment loss in at least one site during the three month monitoring period (AL) and another sub-group of 6 patients who exhibited no attachment loss (NAL). Table 3 shows the median titres and mean avidities of the three classes of

Table 3: Baseline titres and avidities of antibodies to *P. gingivalis* in control subjects, total periodontitis patients and periodontitis patients grouped according to attachment loss and no attachment loss.

	CONTROLS (n=11)		PERIODONTITIS PATIENTS					
			Total (n=20)		Attachment Loss (n=14)		No attachment Loss (n=6)	
	Median Titre (EU)	Mean Avidity (M)	Median Titre (EU)	Mean Avidity (M)	Median Titre (EU)	Mean Avidity (M)	Median Titre (EU)	Mean Avidity (M)
IgG	320 (107-884)*	1.21 (0.85-1.70)	2390 (1017-5595)*	1.74 (1.12-2.14)	2390 (841-4806)	1.66 (1.21-2.04)	2149 (1017-7835)	1.93 (0.95-3.34)
IgM	537 (188-2170)	0.81 (0.69-0.94)	206 (105-603)	0.78 (0.64-0.93)	280 (105-761)	0.70§ (0.60-0.84)	153 (72-722)	0.97§ (0.86-1.07)
IgA	28 (19-60)¶	1.35 (0.60-1.74)	586 (151-1478)¶	1.66 (1.34-1.99)	631 (44-2261)	1.66 (1.16-2.01)	268 (187-14154)	1.66 (1.37-2.04)

*,¶,§ Significant differences between compared groups are shown by paired symbols ($p<0.01$). Interquartile ranges (Q_1 - Q_3) given in parentheses.

Table 4: Titres and avidities of antibodies to *P. gingivalis* for all periodontitis subjects at baseline and after three months.

	Median Titre (EU)				Mean avidity (M)			
	Baseline	3 months	Mean difference	Interquartile range	Baseline	3 months	Mean difference	Interquartile range
IgG	2390	1824	-566	-603/+1400	1.74	2.02	+0.28	-0.16/+0.73
IgM	206	150	-56	-115/+328	0.78	0.70	+0.08	-0.29/+0.11
IgA	586	410	-176	-194/+441	1.66	1.49	-0.17	-0.51/+0.32

No significant differences were detected between baseline and 3 months for all comparisons: Student's paired t-test.

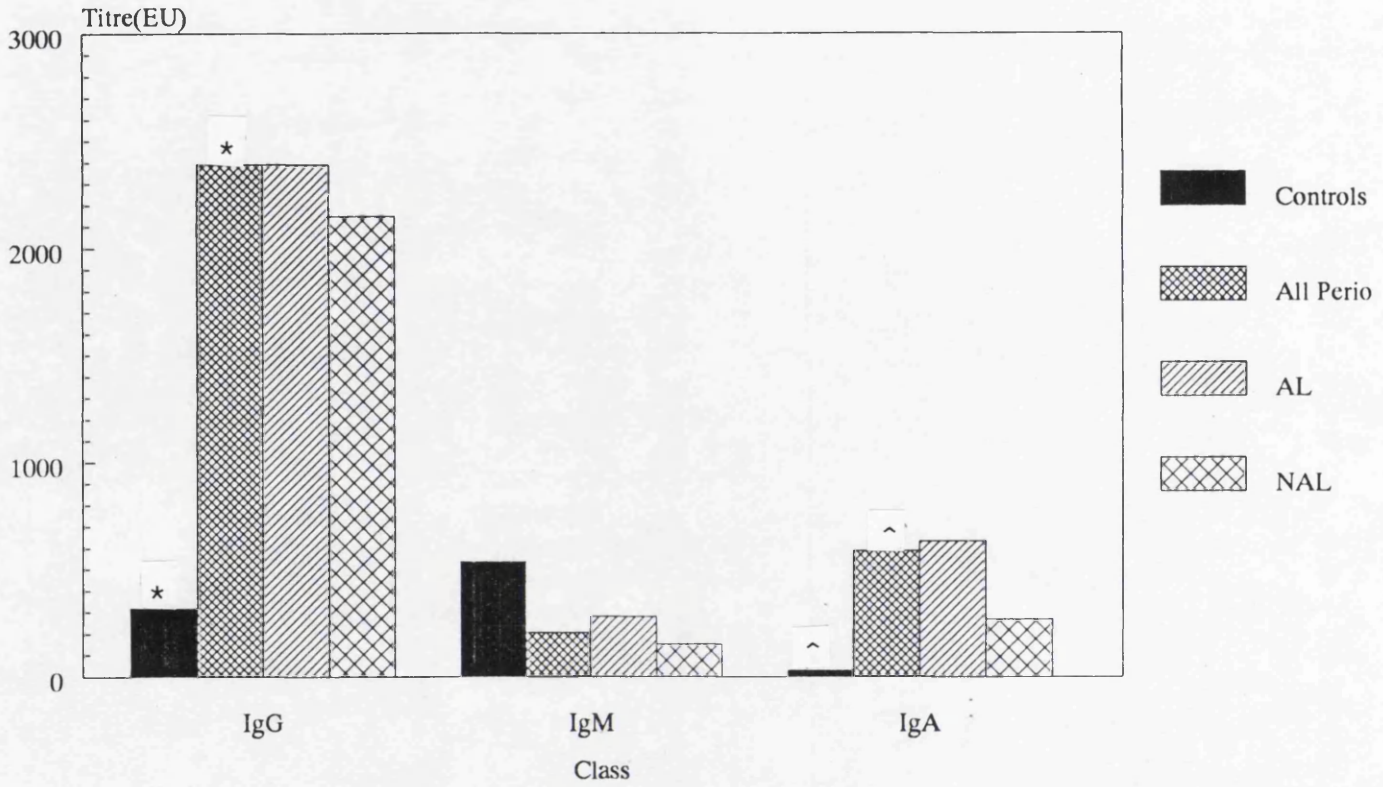

Figure 2: Baseline titres to *P. gingivalis*.

Paired symbols denote significant differences.

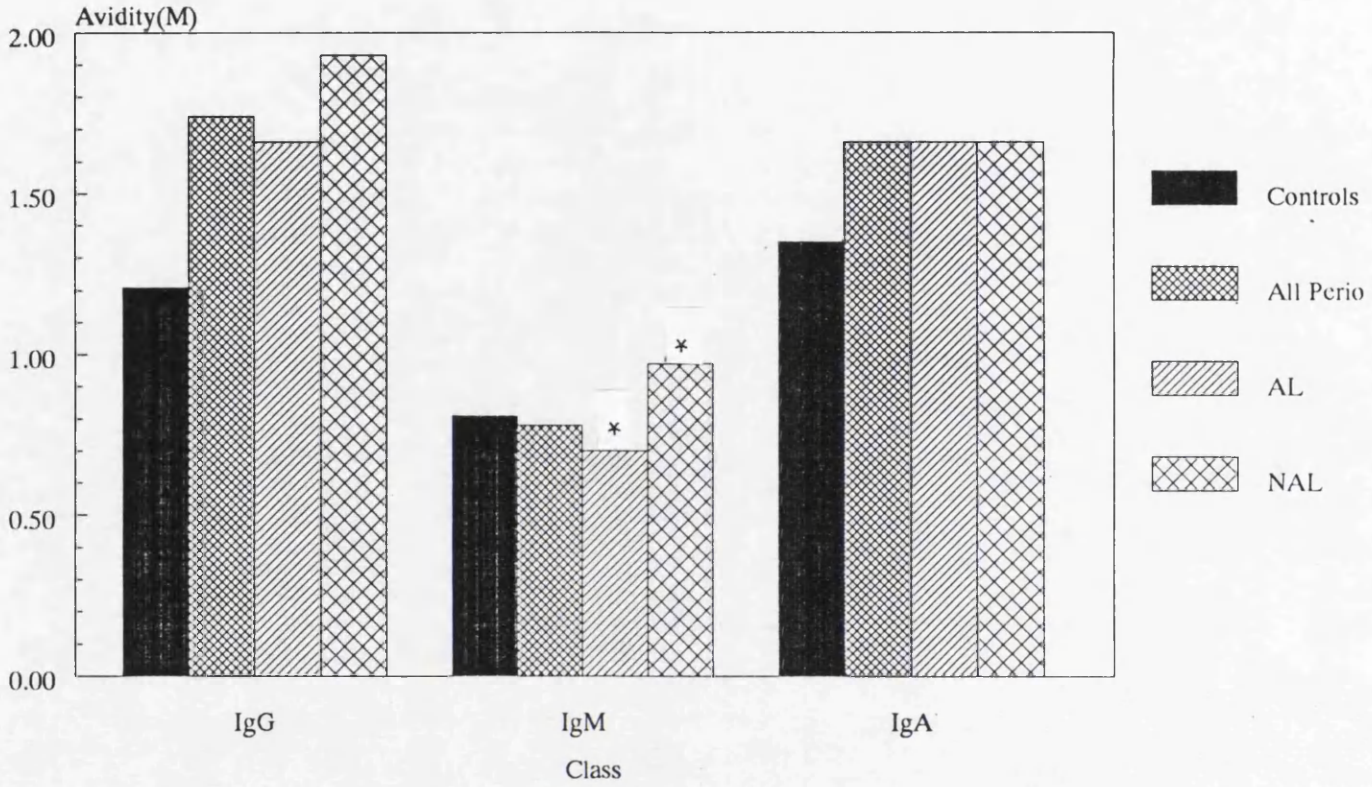

Figure 3: Baseline avidities to *P. gingivalis*.

Paired symbols denote significant differences.

antibodies in controls and periodontitis patients at baseline. Comparisons between patients and controls for titre and avidity to *P. gingivalis* in all three immunoglobulin classes were made and significant differences ($p<0.01$) were only noted between IgG median titres and IgA median titres (Mann-Whitney or two-sample t-test). The comparison between periodontitis patients at baseline grouped in terms of attachment loss and lack of attachment loss after three months is also shown in this table. A significant difference was noted between the mean avidity of IgM antibodies between subjects with attachment loss and those without attachment loss ($p<0.001$). These data are further illustrated in Figures 2 and 3.

Table 4 and Figures 4 and 5 show the changes in antibody titre and avidity between baseline and three months for all periodontitis subjects but indicates no significant differences. Interestingly, sub-grouping the periodontitis patients into AL and NAL gave divergent results for IgG avidity to *P. gingivalis* in that over the three month period, the AL group had IgG avidities which tended to increase whereas the NAL avidities tended to decrease (Table 5 and Figure 6). Similarly, although also not statistically significant, IgM and IgA avidities tended to decrease more in NAL than in AL patients.

Control and periodontitis groups were compared for differences in IgG titre and avidity to the two organisms (*P. gingivalis* and *A. actinomycetemcomitans*) and the results are shown in Table 6. These show that avidities

Table 5: Longitudinal changes in avidities (M) of IgG antibodies to *P. gingivalis* for AL and NAL periodontitis subjects.

		Mean change in avidity (Baseline-3 months)	p-value (Student's paired t-test)
IgG	AL (n=14)	+0.46 (-0.13/+1.12)	0.14
	NAL (n=6)	-0.10 (-0.47/+0.42)	0.74
IgM	AL (n=14)	-0.01 (-0.14/+0.11)	0.80
	NAL (n=6)	-0.29 (-0.60/+0.09)	0.22
IgA	AL (n=14)	-0.07 (-0.54/+0.35)	0.71
	NAL (n=6)	-0.15 (-0.74/+0.61)	0.74

Table 6: Titres (EU) and avidities (EU) of IgG antibodies to *P. gingivalis* and *A. actinomycetemcomitans* for control and periodontitis subjects.

	Control (n=11)		Periodontitis (n=20)	
	P. gingivalis	***Aa***	***P.gingivalis***	***Aa***
IgG Median titre	320 (107-884)	173 (141-214)	2390 (1017-5595)	1189 (109-12289)
IgG Mean avidity	1.21 (0.85-1.70)	0.93 (0.73-1.03)	1.74* (1.12-2.14)	0.83* (0.50-0.94)

* Significant difference: $p<0.01$ for comparison (Student's paired t-test).
Interquartile ranges (Q_1-Q_3) given in parentheses.

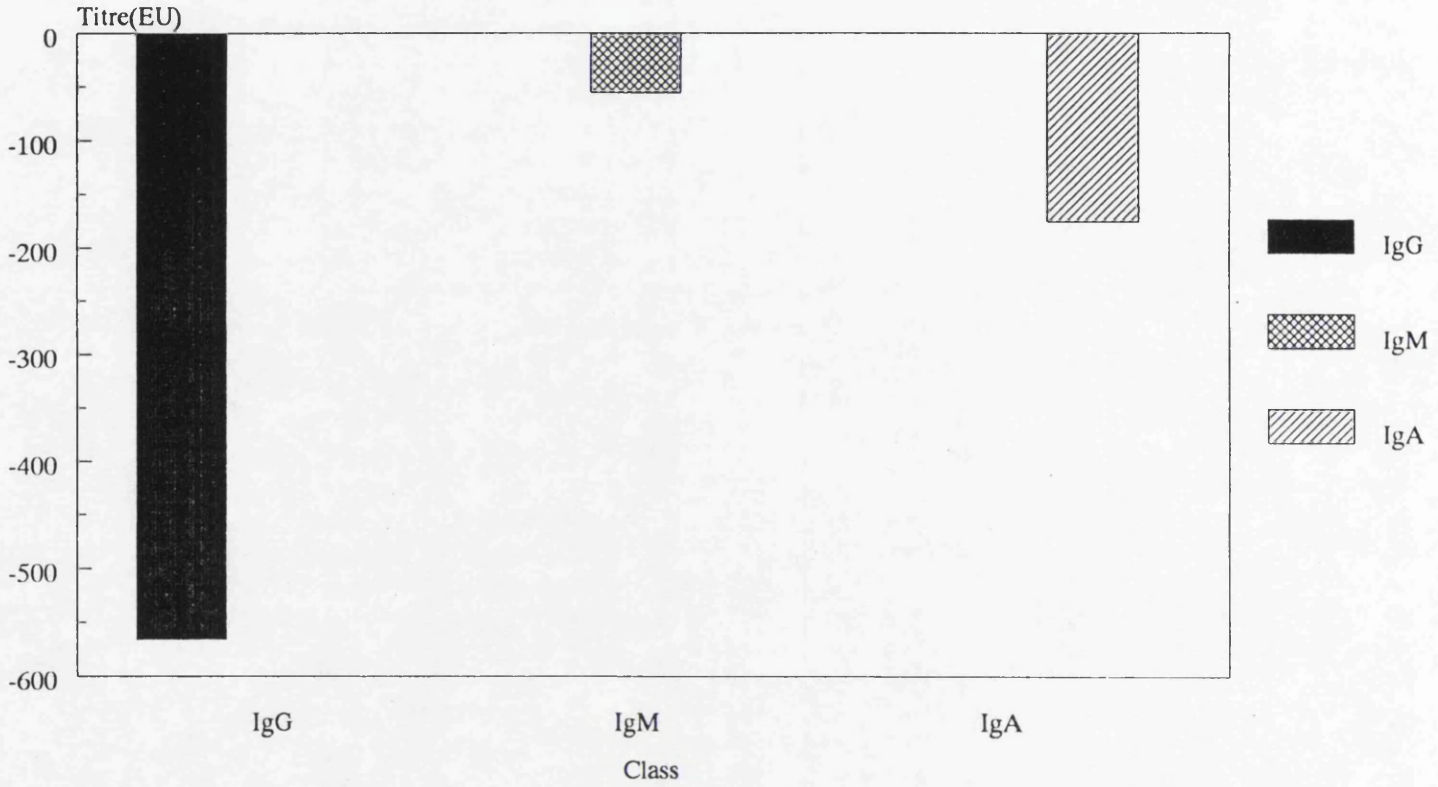

Figure 4: Differences in titres to *P. gingivalis*. Baseline to 3 months (all periodontitis subjects).

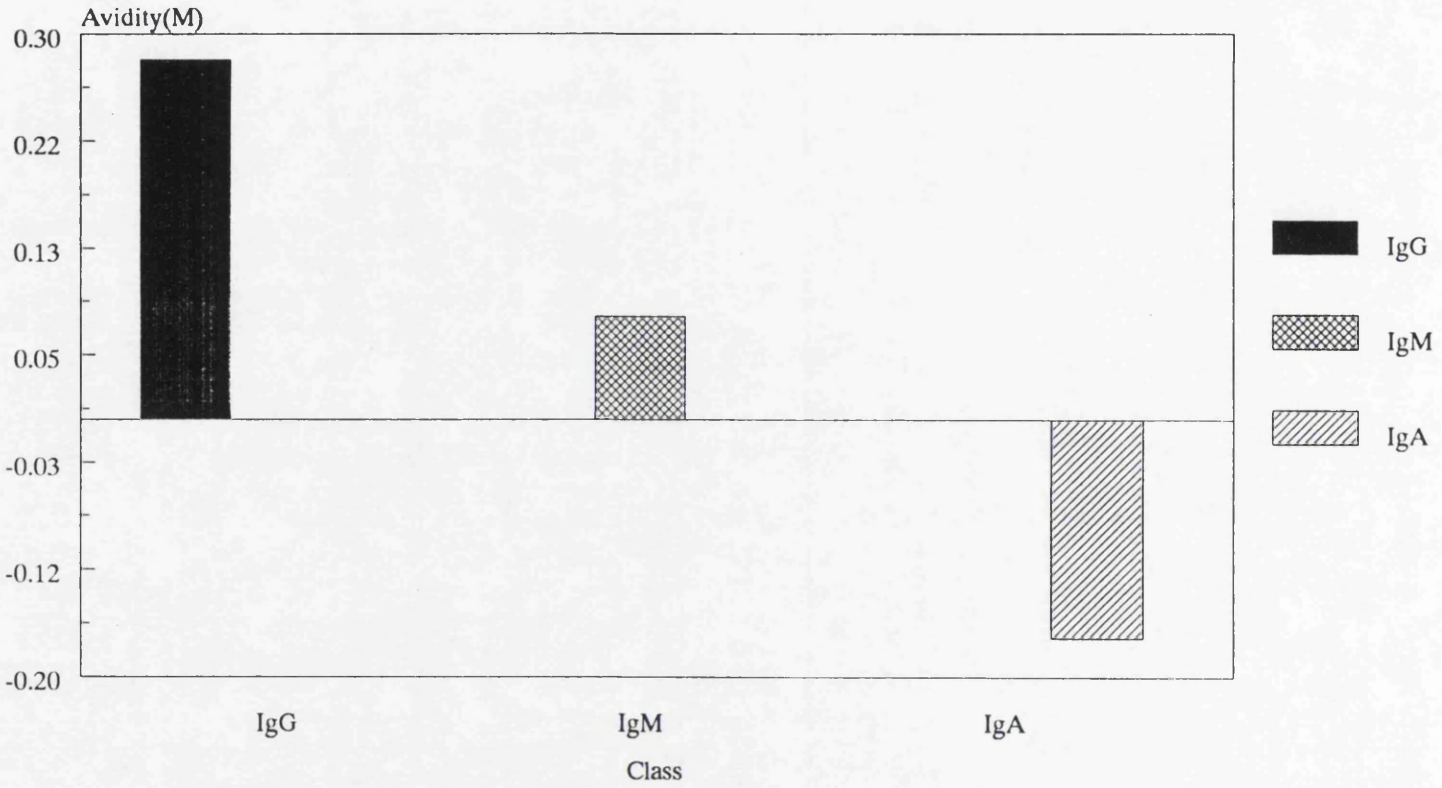

Figure 5: Differences in avidities to *P. gingivalis*. Baseline to 3 months (all periodontitis subjects).

to *P. gingivalis* were significantly higher than to *A. actinomycetemcomitans* in both groups but only in the periodontitis group was this difference significant.

Table 7 presents the correlations between titre and avidity. These correlations are shown for IgG, IgM and IgA specific for *P. gingivalis* and IgG specific for *A. actinomycetemcomitans* (Aa). The subject groups for which these are shown are all subjects, control subjects, all periodontitis subjects, attachment loss periodontitis subjects (AL) and non-attachment loss periodontitis subjects (NAL). The results are shown as R^2 (%) and p-value.

The data presented in Table 7 show that there was a significant correlation between titre and avidity of IgG against *P. gingivalis* for NAL patients and between titre and avidity of IgM against *P. gingivalis* for AL patients.

Table 7: Correlations between titres (EU) and avidities (M) of IgG, IgM and IgA antibodies to *P. gingivalis* and IgG antibodies to *A. actinomycetemcomitans*.

	P. gingivalis			*Aa*
	IgG	**IgM**	**IgA**	**IgG**
All subjects	42.0%[1], <0.001[2]*	10.2%, 0.079	7.5%, 0.257	24.3%, 0.009*
Control subjects	24.3%, 0.123	16.5%, 0.215	0.1%, 0.939	-1.2%, 0.762
All periodontitis subjects	39.2%, 0.003*	12.2%, 0.131	1.4%, 0.689	32.2%, 0.018*
AL	13.7%, 0.193	54.9%, 0.002*	14.1%, 0.284	90.1%, <0.001*
NAL	69.3%, 0.04*	12.6%, 0.491	18.2%, 0.474	0.3%, 0.932

[1] = % R^2,
[2] = p-value, * denotes significant correlations (significance level of $p<0.05$).

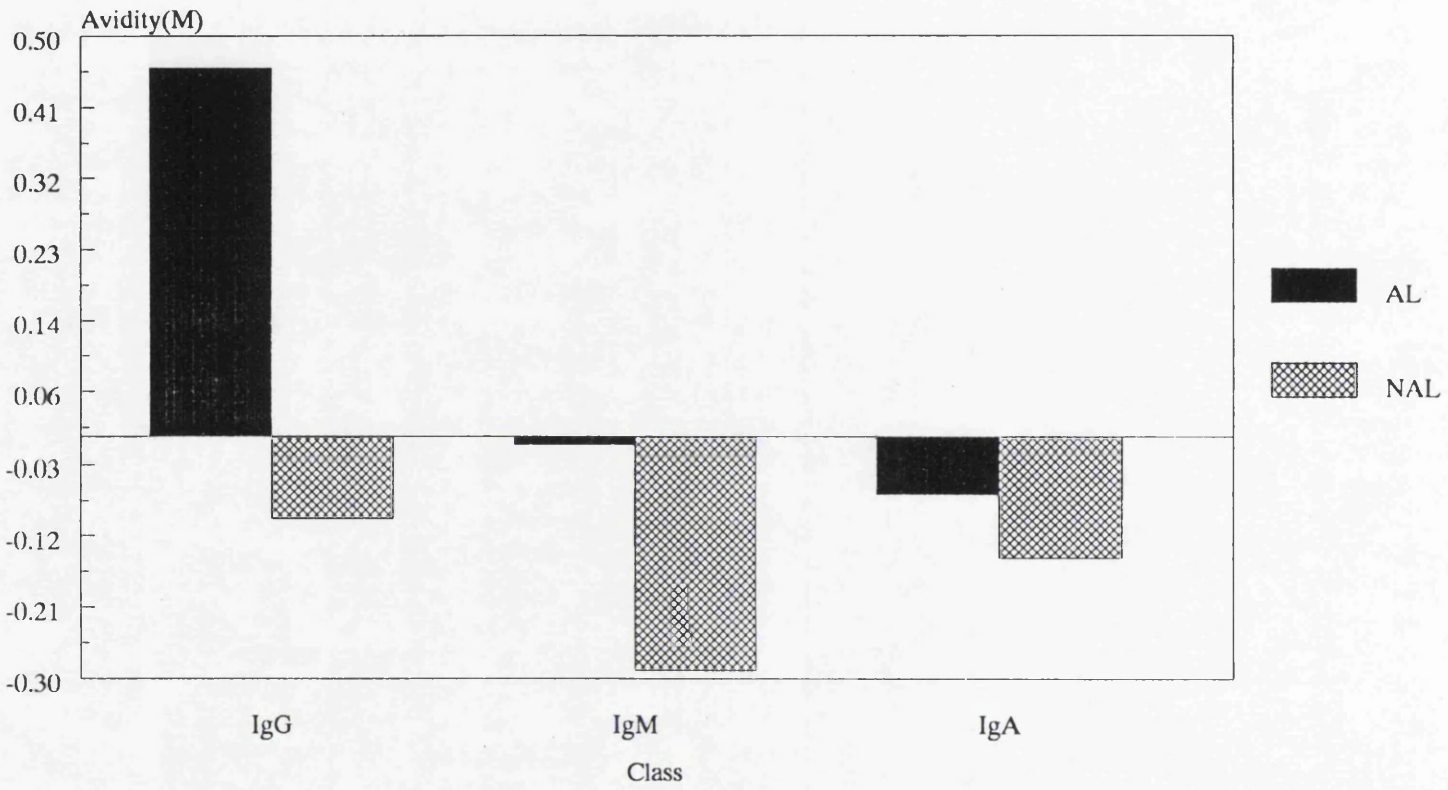

Figure 6: Longitudinal changes in avidity to *P. gingivalis*. Baseline to 3 months (AL and NAL subjects).

3.1.3. Comparison of antibody avidity in various forms of periodontal disease

The relationships between various forms of periodontal disease and the avidities of serum antibodies of all three immunoglobulin classes (IgG, IgM and IgA) to *P. gingivalis* and *A. actinomycetemcomitans* were investigated. Twenty-four patients with untreated adult periodontitis (AP) and twelve patients diagnosed as suffering from the early-onset form of periodontitis, rapidly progressive periodontitis (RPP) were studied. The latter group were age and sex matched to healthy controls (C).

The differences between median avidity and median titre of antibody to both organisms for AP and RPP groups are given in Tables 8 and 9 and also in Figures 7-10. These show that avidity of IgG tended to be higher and IgM avidity to *P. gingivalis* was significantly higher in AP than in RPP patients, and that IgA titre was significantly lower in RPP patients. In contrast, IgA avidity to *A. actinomycetemcomitans* tended to be higher in RPP than in AP and IgG and IgA titres were significantly higher in RPP than in AP. Statistically significant differences between groups were assessed using the Mann-Whitney U-test and p-values are given. Similarly, the differences between median avidity of antibody to both organisms for AP and seronegative RPP groups are given in Table 10. Statistically significant differences between groups were assessed using the Mann-Whitney U-test and p-values are

Table 8: Median avidities and titres of antibodies to *P. gingivalis* for AP and RPP groups. Where significant, values for Mann-Whitney U-tests are given.

	P. gingivalis					
	Avidity (M)			Titre (EU)		
	IgG	IgM	IgA	IgG	IgM	IgA
AP (n=24)	0.92	0.74	1.15	653	357	80
p-value	0.065(NS)	<0.001				0.025
RPP (n=12)	0.58	0.54	1.00	524	206	11

Table 9: Median avidities and titres of antibodies to *A. actinomycetemcomitans* for AP and RPP groups. Where significant, values for Mann-Whitney U-tests are given.

	A. actinomycetemcomitans					
	Avidity (M)			Titre (EU)		
	IgG	IgM	IgA	IgG	IgM	IgA
AP (n=24)	**0.65**	**0.61**	**0.80**	**319**	**545**	**10**
p-value			**0.065(NS)**	**<0.01**		**0.035**
RPP (n=12)	**0.74**	**0.61**	**1.08**	**4810**	**301**	**144**

Table 10: Mean avidities of IgG antibodies to *A. actinomycetemcomitans* and *P. gingivalis* for AP and seronegative RPP groups. Where significant, values for two-sample t-tests are given.

	IgG Avidity (M)	
	P. gingivalis	***A. actinomycetemcomitans***
AP (n=24)	**0.92**	**0.65**
p-value	**0.004**	
RPP (n=10)	**0.53**	**0.70**

Table 11: Mean avidities of IgG antibodies to *P. gingivalis* for seronegative RPP patients and their age/sex matched controls. Paired t-tests result is given.

	P. gingivalis
	Avidity (M)
	IgG
Seronegative RPP (n=10)	**0.53**
p-value	**0.26(NS)**
Controls (n=10)	**0.76**

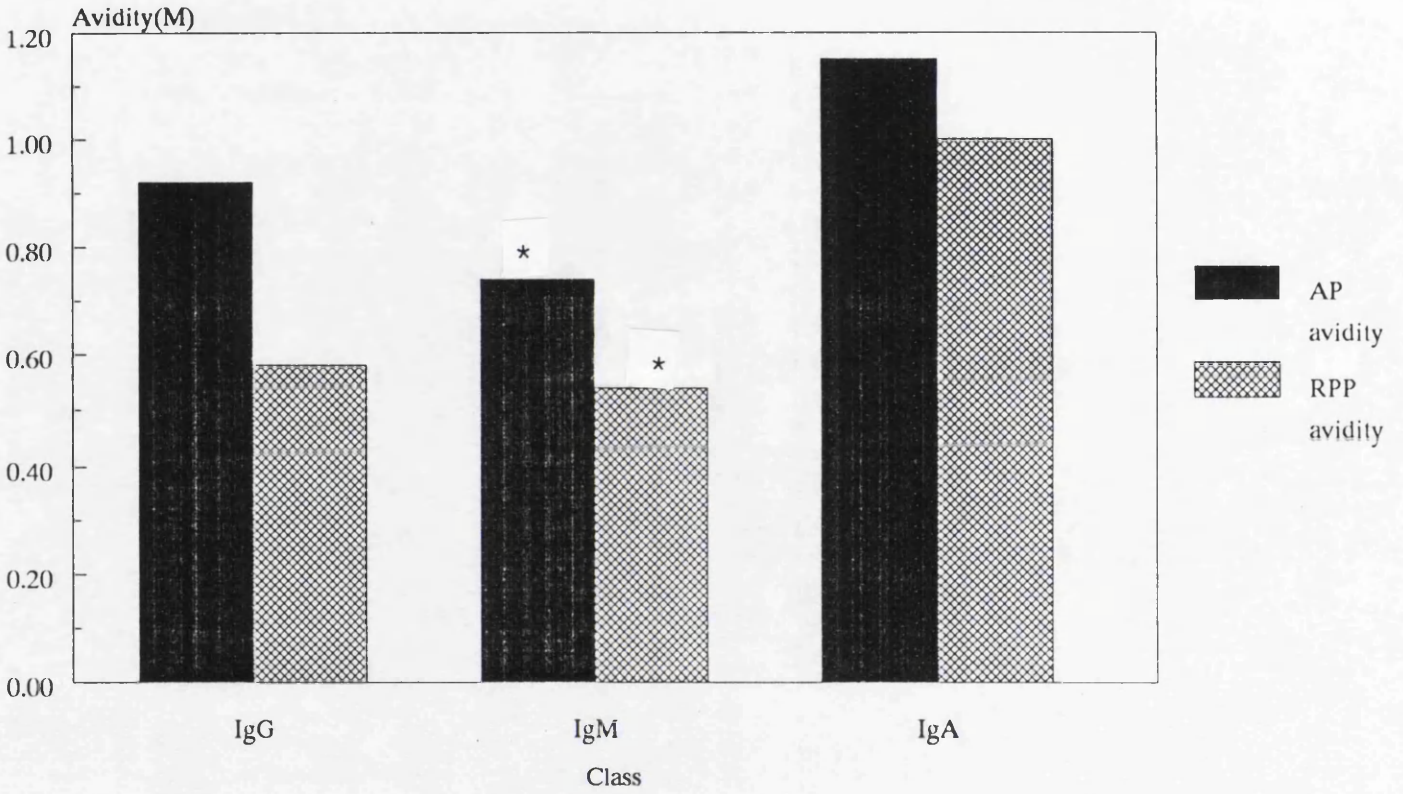

Figure 7: Differences between AP and RPP in terms of avidity to *P. gingivalis*.

Paired symbols denote significant differences.

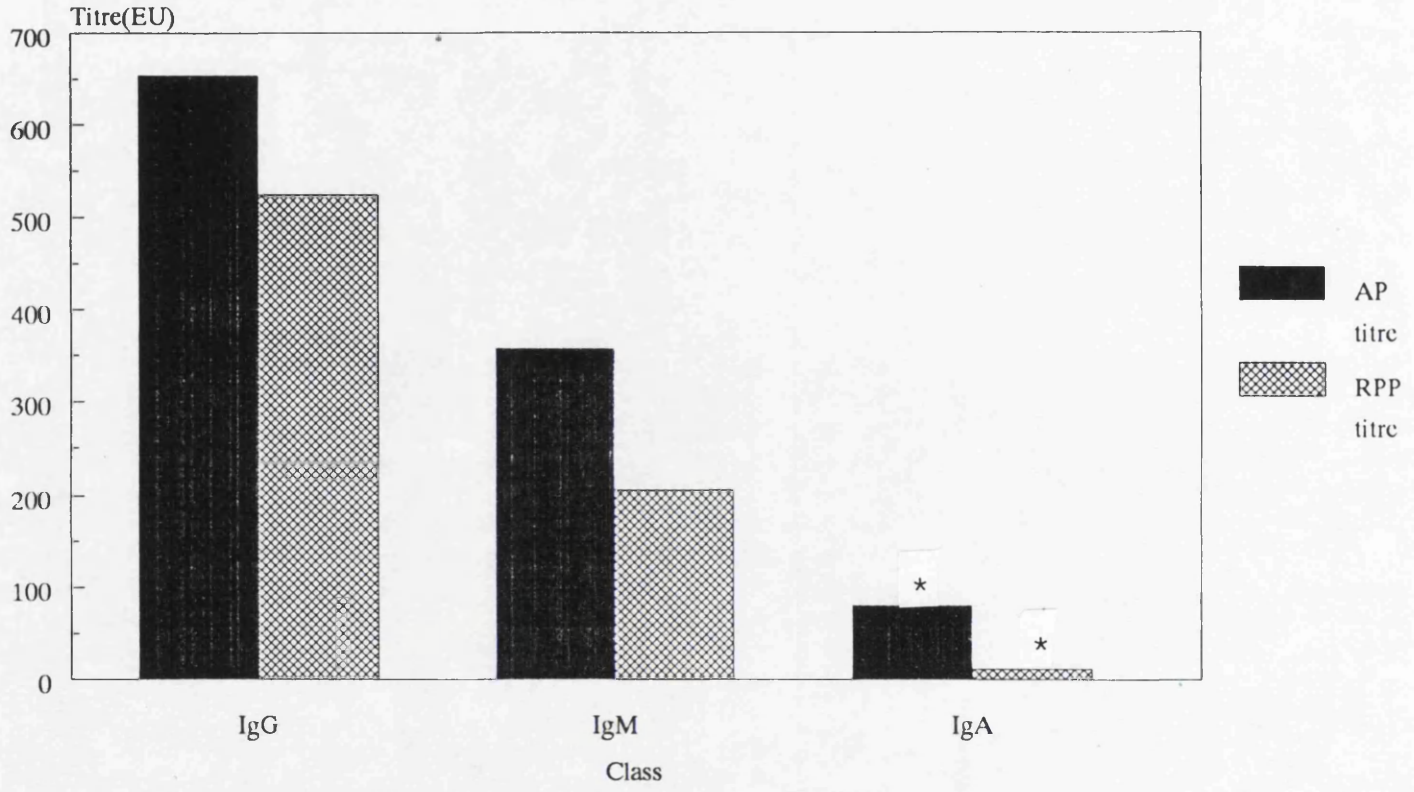

Figure 8: Differences between AP and RPP in terms of titre to *P. gingivalis*.

Paired symbols denote significant differences.

given. Seronegativity here is as defined by Chen *et al* (157), and it should be noted that the seronegative groups for *P. gingivalis* and *A. actinomycetemcomitans* were not identical, i.e. two patients were seropositive for *P. gingivalis* and another two for *A. actinomycetemcomitans*.

A comparison between seronegative RPP patients and their age/sex matched controls in terms of IgG avidity to *P. gingivalis* is shown in Table 11 and a paired t-test was used.

The percentage of patients in both patient groups who were seropositive in terms of titre and avidity to *P. gingivalis* and *A. actinomycetemcomitans* are shown in Tables 12 and 13. The seropositivity definition is extended here to include avidity, i.e. avidity > 2x median control avidity.

The results of a discriminant analysis of AP and RPP groups are given in Table 14. This was performed with cross-validation thereby mimicking a prospective analysis. Cross-validation operates by removing each point in turn and treating it as an unknown value, comparing its resultant predicted value with its true value. Data are shown for the analyses of the whole RPP group and also the seronegative RPP group.

Correlations between titre and avidity of antibody to *P. gingivalis* and *A. actinomycetemcomitans* are given in Table 15 for AP, RPP and control (C) groups. Results are presented as R^2 and p-value, if significant.

Table 12: Percentage of patients seropositive in terms of titre and avidity of antibody to *P. gingivalis* for AP and RPP groups. Seropositivity defined as > 2x control median.

	P. gingivalis					
	Avidity			**Titre**		
	IgG	IgM	IgA	IgG	IgM	IgA
AP (n=24)	**17**	**0**	**12**	**42**	**25**	**67**
RPP (n=12)	**17**	**0**	**8**	**25**	**25**	**33**

Table 13: Percentage of patients seropositive in terms of titre and avidity of antibody to *A. actinomycetemcomitans* for AP and RPP groups. Seropositivity defined as > 2x control median.

	A. actinomycetemcomitans					
	Avidity			**Titre**		
	IgG	**IgM**	**IgA**	**IgG**	**IgM**	**IgA**
AP (n=24)	**0**	**12**	**0**	**17**	**67**	**42**
RPP (n=8)	**17**	**25**	**17**	**50**	**58**	**83**

Table 14: Discriminant analysis of AP and RPP groups with cross-validation for entire RPP group and seronegative RPP group.

	True group	
Assigned group	**AP (n=24)**	**RPP (n=12) (n=10 for seronegative group)**
AP	**11**	**3**
RPP	**13**	**9**
% correct	**46**	**75**
AP	**14**	**1**
sero-negative RPP	**10**	**9**
% correct	**58**	**90**

Table 15: Correlations between titres and avidities of IgG, IgM and IgA antibodies to *P. gingivalis* and *A. actinomycetemcomitans* for AP, RPP and C groups.

	P. gingivalis			*A. actinomycetemcomitans*		
	IgG	**IgM**	**IgA**	**IgG**	**IgM**	**IgA**
AP	19.2%[1], 0.03[2]*	1.2%	36.4%, 0.002*	6.8%	73.8%, 0.003*	0.1%
RPP	81.6%, <0.001*	36.1%, 0.04*	3.1%	80.5%, <0.001*	1.4%	36.4%, 0.04*
C	1.5%	24.0%	9.1%	14.4%	61.2%, 0.003*	9.6%

[1] = R^2 (%),
[2] = p-value, * denotes significant correlations (significance level of $p<0.05$).

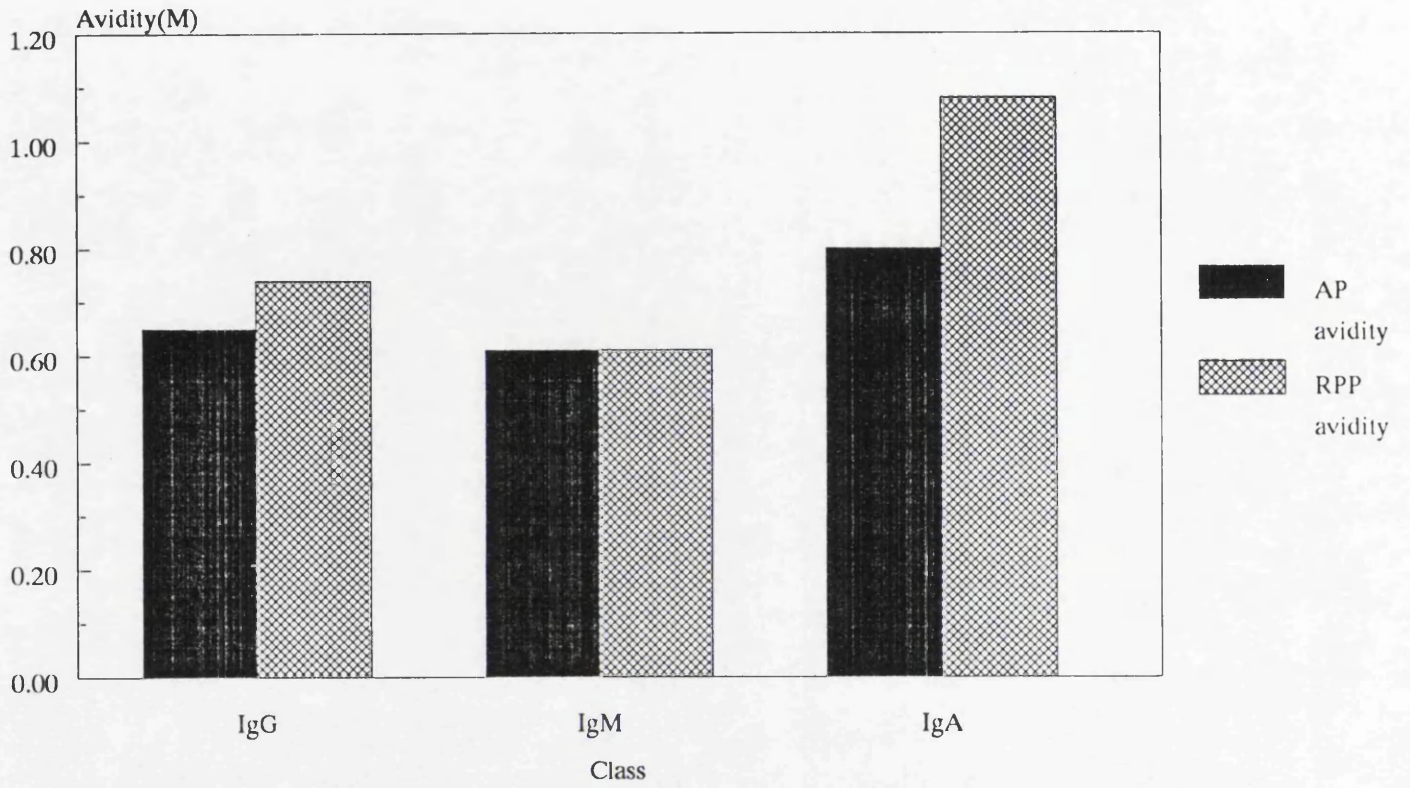

Figure 9: Differences between AP and RPP in terms of avidity to *A. actinomycetemcomitans*.

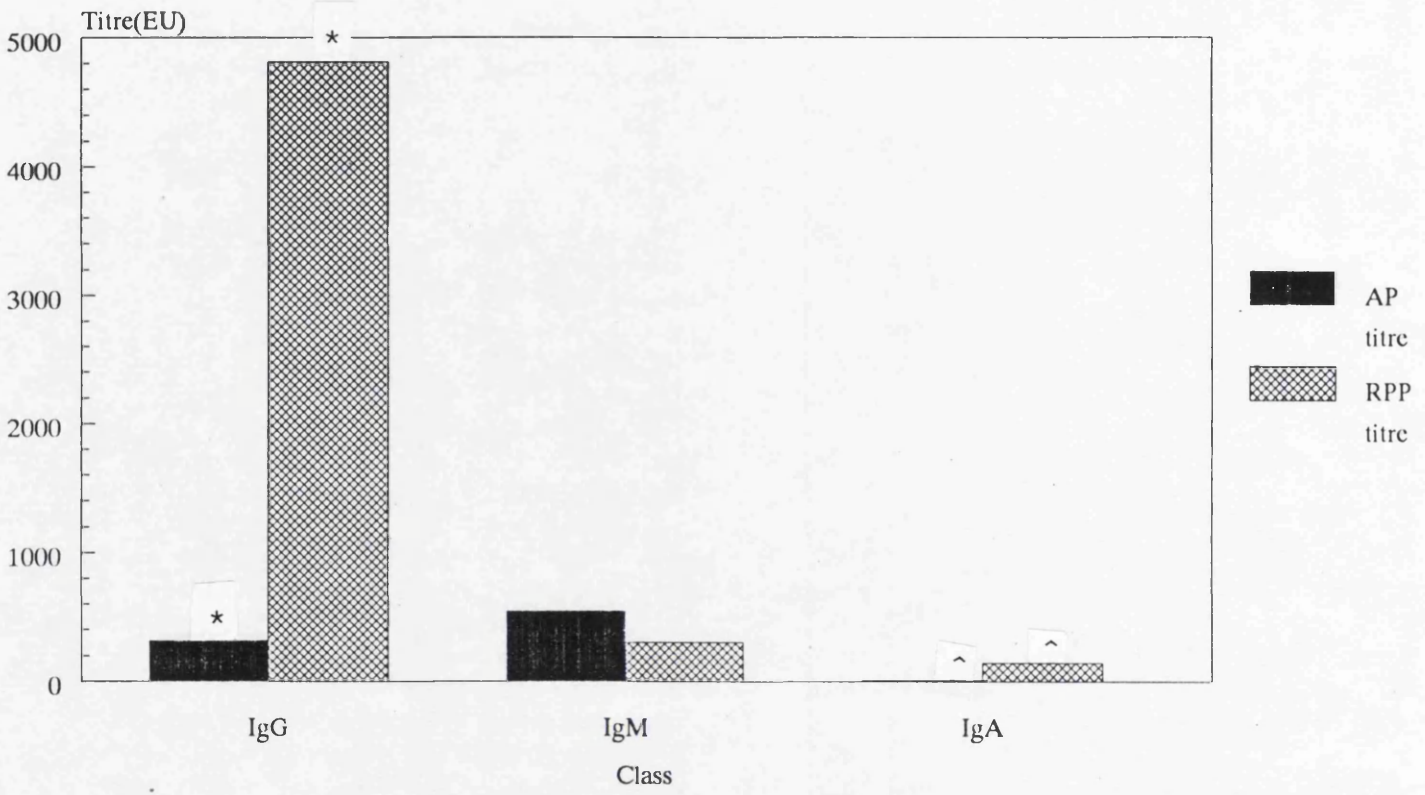

Figure 10: Differences between AP and RPP in terms of titre to **A.** *actinomycetemcomitans.*

Paired symbols denote significant differences.

3.1.4. Investigation of treatment effect on systemic antibody

This longitudinal study of nineteen adult periodontitis patients investigated IgG, IgM and IgA antibody titres and avidities to *P. gingivalis* and *A. actinomycetemcomitans* before and after periodontal therapy. These data are given in Tables 16-19. A significant increase in avidity of IgG antibody against *P. gingivalis* was observed between baseline and post-treatment (Table 16 and Figure 11), whereas the titre of IgA antibody to this organism increased significantly (Table 17 and Figure 12). There were no significant differences in the avidities of any class to *A. actinomycetemcomitans* during the monitoring period (Table 18 and Figure 13). However, the titre of all three classes to *A. actinomycetemcomitans* increased significantly post-therapy (Table 19 and Figure 14).

Table 16: Mean avidity (M) of antibodies against *P. gingivalis*.

	Baseline	Post-treatment	p-value
IgG	0.97	1.35	0.03
IgM	0.75	0.97	0.16
IgA	1.25	1.20	0.80

p-value given for t-test using null-hypothesis.

Table 17: Median titre (EU) of antibodies against *P. gingivalis*.

	Baseline	Post-treatment	p-value
IgG	654	1346	0.45
IgM	380	479	0.33
IgA	88	226	0.003

p-value given for t-test using null-hypothesis (log transformed data).

Table 18: Mean avidity (M) of antibodies against *A. actinomycetemcomitans*.

	Baseline	Post-treatment	p-value
IgG	0.79	0.66	0.30
IgM	0.48	0.53	0.33
IgA	0.87	0.80	0.50

p-value given for t-test using null-hypothesis

Table 19: Median titre (EU) of antibodies against *A. actinomycetemcomitans*.

	Baseline	Post-treatment	p-value
IgG	104	650	0.006
IgM	230	497	0.02
IgA	9	18	0.008

p-value given for t-test using null-hypothesis (log transformed data).

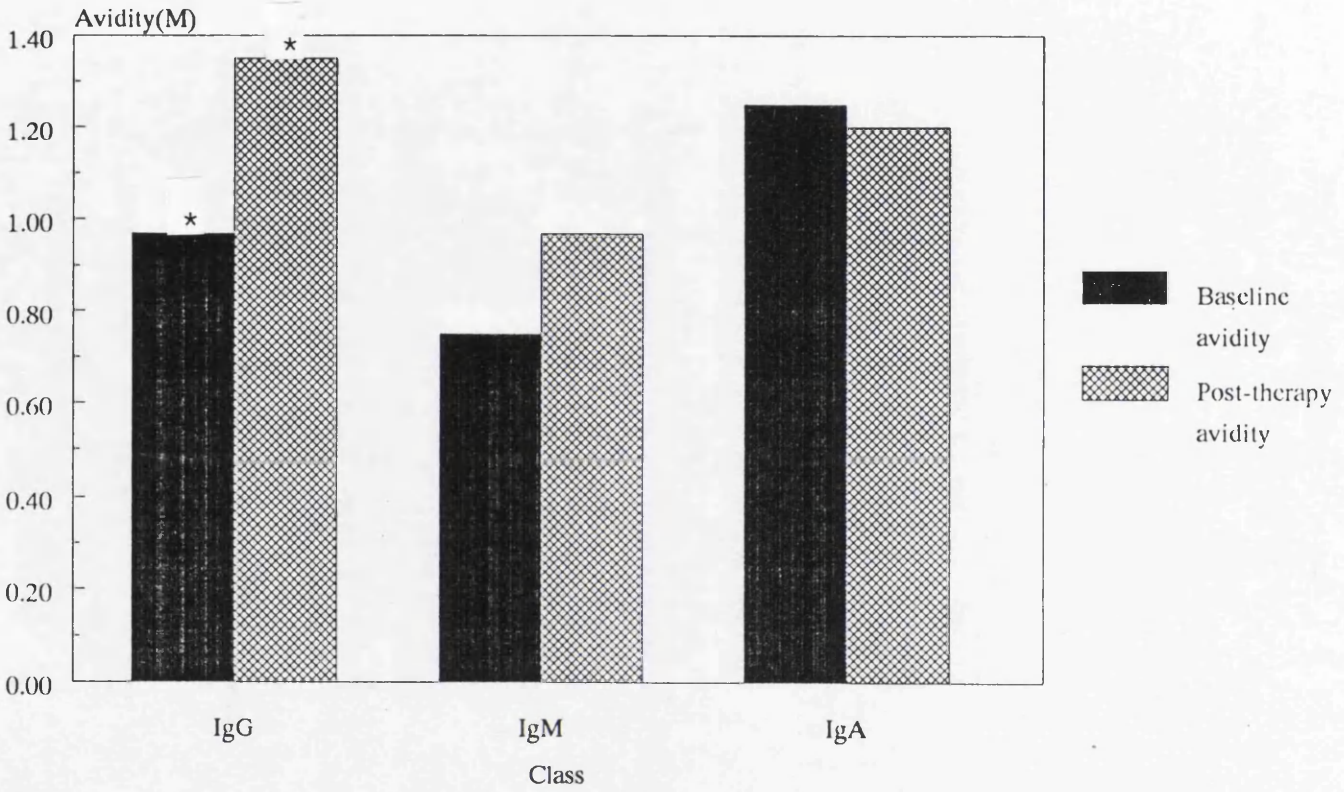

Figure 11: Differences between baseline and post-therapy in terms of avidity to *P. gingivalis*.

Paired symbols denote significant differences.

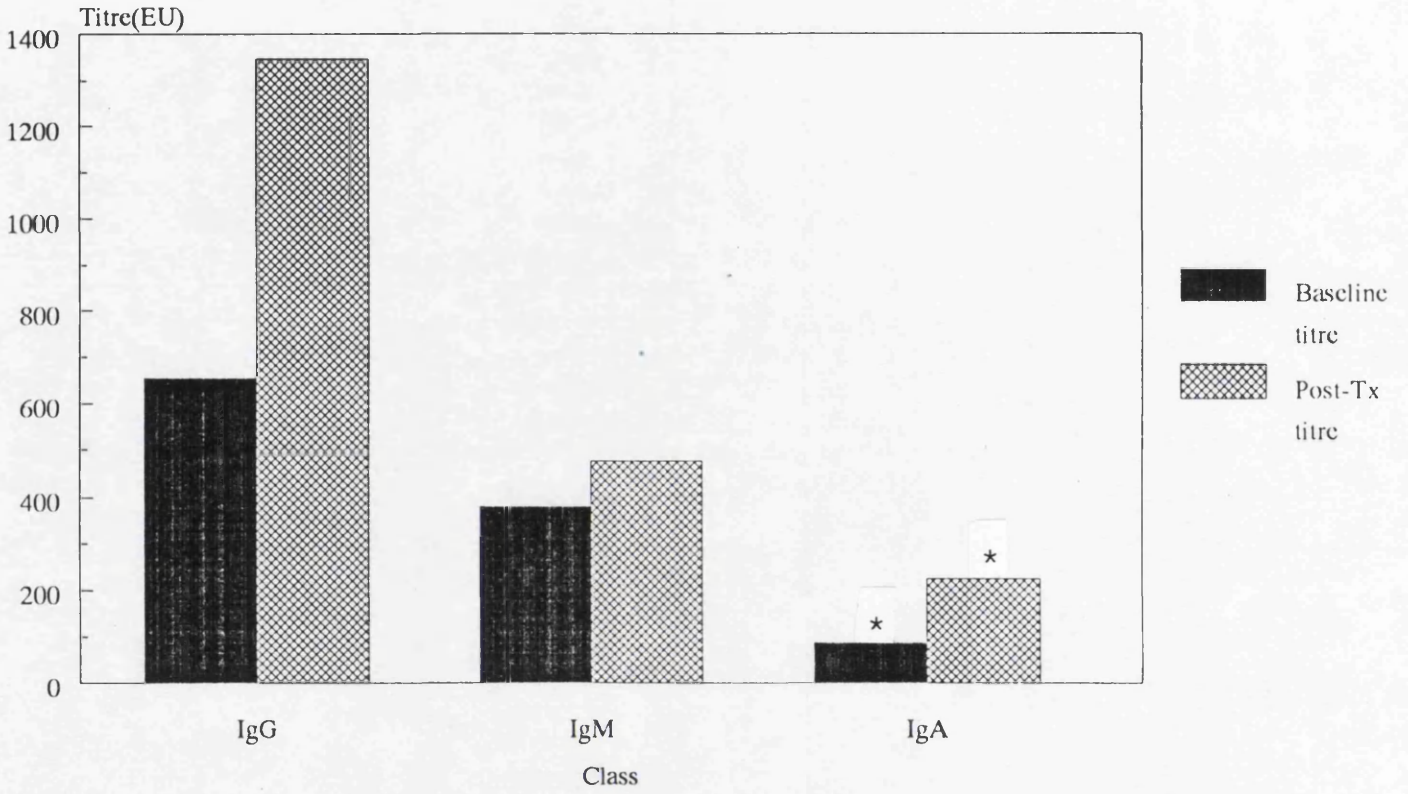

Figure 12: Differences between baseline and post-therapy in terms of titre to *P. gingivalis*.

Paired symbols denote significant differences.

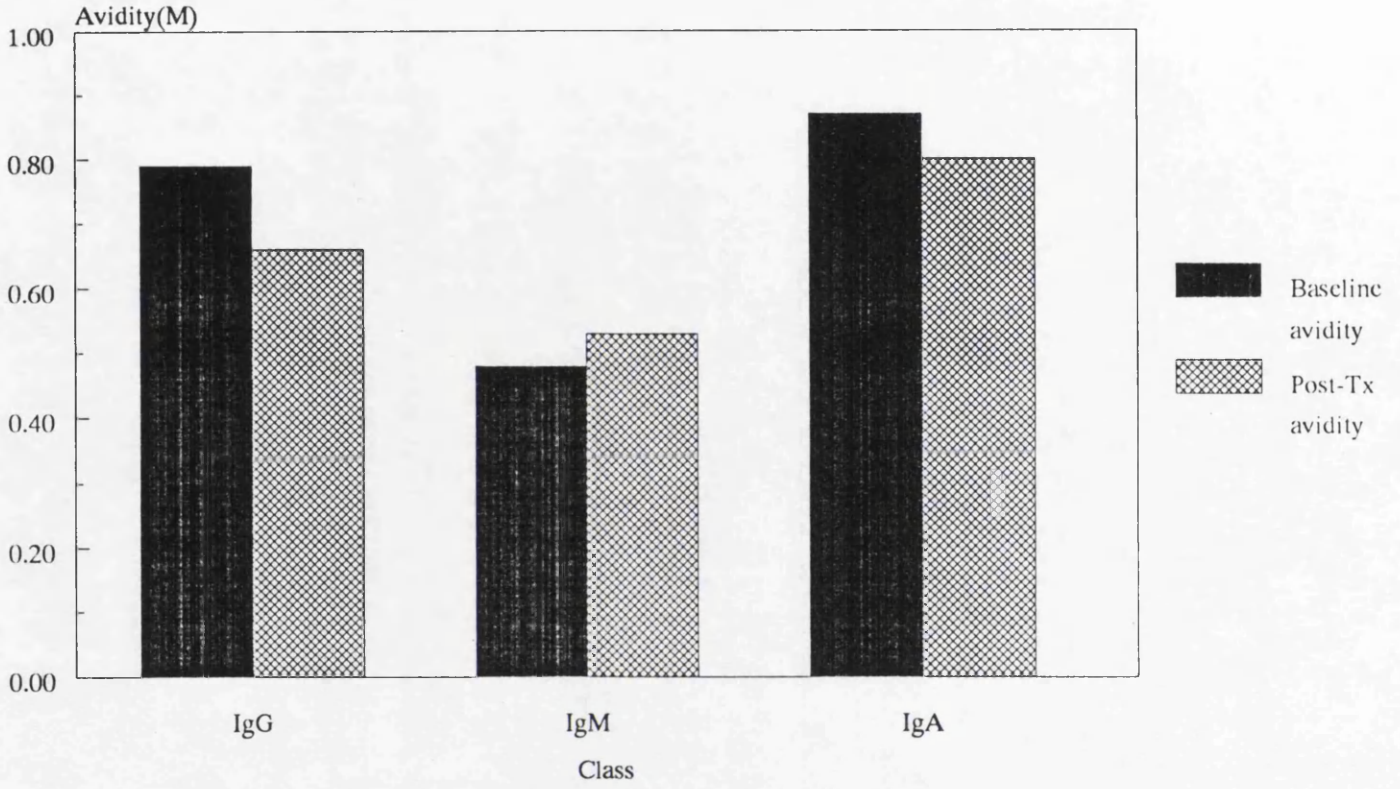

Figure 13: Differences between baseline and post-therapy in terms of avidity to *A. actinomycetemcomitans*.

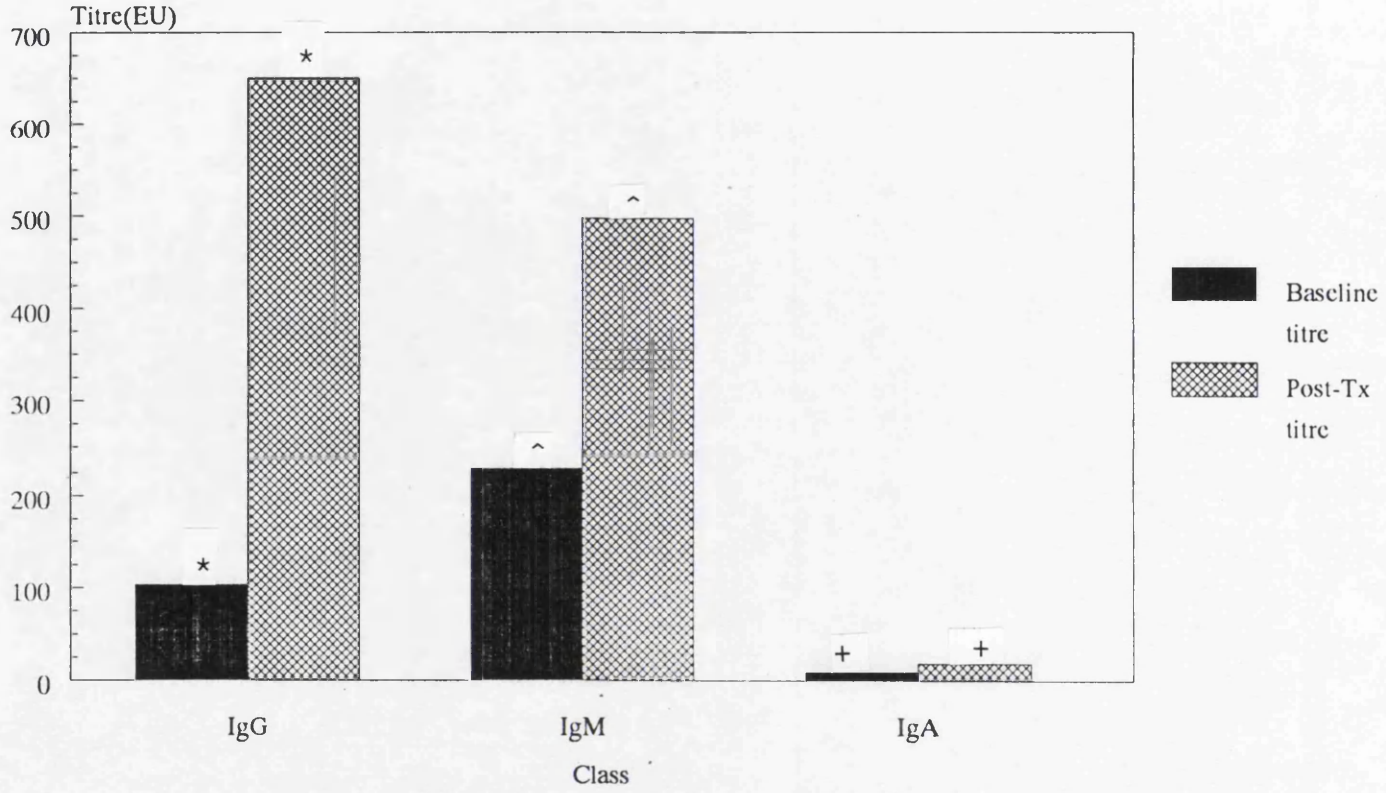

Figure 14: Differences between baseline and post-therapy in terms of titre to *A. actinomycetemcomitans*.

Paired symbols denote significant differences.

3.2. Local Response

3.2.1. Comparison of local antibody titres in adult periodontitis patients (sites with differing clinical indices)

Specific IgG, IgA and IgM antibody titres to *P. gingivalis* and *A. actinomycetemcomitans* were measured by ELISA in serum and gingival crevicular fluid (GCF) at five sites in each of twenty chronic periodontitis patients. Specific serum antibody titres correlated with mean gingival crevicular fluid titres ($p<0.001$). The three immunoglobulin subclass responses (IgA, IgG and IgM) to *P. gingivalis* correlated (R^2 ranged from 24.4% to 70.9% and in all cases $p<0.03$). A comparison of sites with PD<4mm and PD≥4mm showed that the latter group had significantly lower gingival crevicular fluid IgG titres to *P. gingivalis* ($p<0.01$). Sites with GI=3 had significantly lower gingival crevicular fluid IgG titres to this organism than those with GI<3 ($p<0.001$).

The Mann-Whitney tests relating the sites grouped on pocket depth and gingival index with local IgG antibody levels to *P. gingivalis* and *A. actinomycetemcomitans* are presented in Table 20. *P. gingivalis* IgG levels were found to be significantly lower in the sites with pocket depth greater than or equal to 4mm as compared with PD<4 groups ($p<0.01$) and GI=3 as compared with GI<3 groups

Table 20: Comparisons between different degrees of PD and GI and IgG antibodies in GCF to *P. gingivalis* and *A. actinomycetemcomitans* at these sites (Mann-Whitney test).

	P. gingivalis IgG (EU/30s)			*A. actinomycetemcomitans* IgG (EU/30s)		
	n	mean	(SD)	n	mean	(SD)
PD<4	45	891*	(2,304)	17	25[1]	(46)
PD≥4	49	518*	(1,775)	32	21[1]	(46)
GI<3	61	1,602+	(2,465)	36	21[2]	(43)
GI=3	28	24+	(67)	8	41[2]	(64)

* p<0.01 p-values for comparison of paired values,
+ p<0.001 p-values for comparison of paired values,
[1], [2] p<0.05 i.e. Not Significant.
(a) Comparisons for all sites were not performed due to the limited volume of gingival crevicular fluid available in site samples.
(b) IgA response to P. gingivalis was also tested but gave no significant differences between clinical index groupings.

Table 21: Correlations between serum and mean GCF antibodies.

	subjects (n)	Correlation Coefficient	Serum mean (EU/ml)	GCF mean (EU/ml)	R^2	p-value
Pg IgG	16	+0.816	2,987	512	66.6%	<0.001
Pg IgA	13	+0.216	265	157	4.7%	NS
Aa IgG	10	+0.982	2,738	121	96.4%	<0.001

Aa = *Actinobacillus actinomycetemcomitans*;
NS = Not Significant;
Pg = *Porphyromonas gingivalis*.
N.B. correlations of Pg IgM and Aa IgM and IgA were not performed due to the limited volume of gingival crevicular fluid available in site samples.

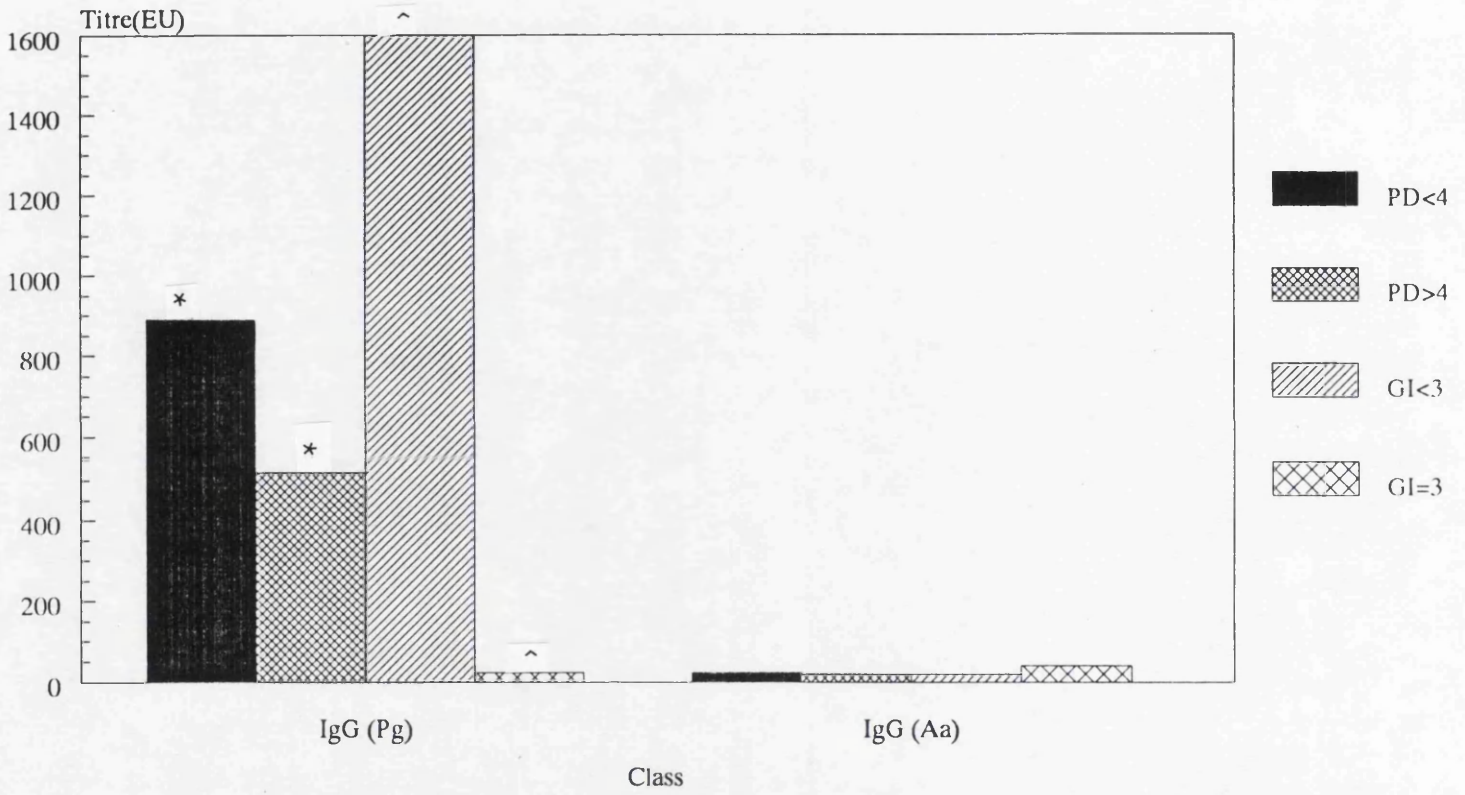

Figure 15: Comparison between clinical index groups in terms of titre to *P. gingivalis* and *A. actinomycetemcomitans*.

Paired symbols denote significant differences.

($p<0.001$). Further tests relating the sites grouped on pocket depth and gingival index with IgG antibody to *A. actinomycetemcomitans* (Table 20) or IgA antibodies to *P. gingivalis* gave no statistically significant differences for these comparisons. The data are further illustrated in Figure 15. Table 21 shows the correlations between serum and mean GCF levels for IgG and IgA against *P. gingivalis* and IgG directed against *A. actinomycetem-comitans*. Both *P. gingivalis* and *A. actinomycetem-comitans* IgG serum and GCF levels correlated well (R^2 = 66.6% and 96.4% respectively; $p<0.001$). Reanalysis of the data categorised on clinical indices revealed slightly higher R^2 values for the sites with more disease (i.e. GI = 3 and PD ≥ 4mm) but did not show a significant trend. Due to the restrictions imposed by the limited sample volume (mean = 0.31µl) obtained using the standardised 30s gingival crevicular fluid sampling technique, insufficient sample was available to compare, via ELISA, specific gingival crevicular fluid IgM with IgA and IgG. Previous work within this laboratory has indicated that levels of total and specific IgM in gingival crevicular fluid were much lower than both IgA and IgG.

Table 22 shows the Spearman rank correlations between PD, GI and IgG antibody to *P. gingivalis*. All of these correlations are statistically significant but the relatively low R^2 values suggest that other factors have influence.

Table 22: Statistically significant correlations ($p<0.05$) between PD, GI and serum IgG antibody levels to Porphyromonas gingivalis. R^2 values are given.

	GI	GCF IgG	Serum IgG
PD	+6.7%	-5.4%	-9.5%
GI	-	-13.5%	-7.2%
GCF IgG	-	-	+66.6%

Table 23: Mean GCF volumes (ul) collected from PD<4 and PD>4 sites, and GI<3 and GI=3 sites.

	Mean	SD
PD<4	0.28	0.24
PD>4	0.33	0.24
GI<3	0.27	0.23
GI=3	0.36	0.26

Table 23 shows the mean GCF volumes collected from sites with differing clinical indices. These data are presented to establish that GCF volumes were not lower in more diseased sites; if this were so it would be a possible explanation for the finding that titres are lower in more diseased sites when expressed as EU/30s sample. In fact, there is a trend towards higher GCF yields from more diseased sites.

Table 24 shows the percentage of sites and patients from which suspected periodontopathogens were isolated. No further data are presented from the microbiological aspect of this study, since no correlation could be demonstrated between the isolation of an organism and the titre of antibody to the homologous organism.

Table 24: Percentage of sites and patients from which suspected periodontopathogens were isolated.

Organism	% of sites	% of patients
P. gingivalis	**2**	**5**
A. actinomycetemcomitans	**4**	**10**
H. aphrophilus	**13**	**25**
B. endodontalis	**6**	**10**
P. intermedia	**6**	**25**

3.2.2. Comparison of humoral immunity in GCF and PISF

There were two main aims of this investigation; firstly, to determine the levels of acute-phase proteins and IgG against *P. gingivalis* in peri-implant sulcus fluid (PISF) and their association with the clinical condition of the peri-implant mucosa; and secondly, to compare the inflammatory and immunological responses at implants and teeth as reflected by the gingival crevicular fluid (GCF) and PISF levels of acute-phase proteins and immunoglobulins.

The clinical data are depicted in Table 25. By definition, inflamed implant and tooth sites had higher MGI scores than healthy implant and tooth sites respectively. PI was also higher at both categories of inflamed sites when compared to their healthy counterparts. Average probing depths were deeper at implants when compared to teeth, particularly at healthy sites, and in addition, average GCF volumes were higher. 67% of the healthy implant sites bled on probing compared to 14% of healthy tooth sites. The percentage of bleeding implant and tooth sites, allocated to the inflamed category was 93% and 85% respectively.

Multivariate repeated measures analysis of variance (MANOVA) demonstrated a significant effect when healthy and inflamed tooth sites ($p=0.021$) were compared for their GCF absolute amounts of α2-M, α1-AT, TF, LF, Alb (ng/30s sample) and IgG against *P.gingivalis* (EU/30s). Follow-up

Table 25: Clinical indices at teeth and implants. The average (SD) are given as well as the frequency distribution and percentages for MGI, PI, PD, BOP and GCF.

		Implant sites		Tooth sites	
		Healthy n=31	**Inflamed n=31**	**Healthy n=21**	**Inflamed n=27**
	Score	n (%)	n (%)	n (%)	n (%)
MGI	0	25 (81)	-	17 (81)	-
	1	6 (19)	-	4 (19)	-
	2	-	18 (58)	-	10 (37)
	3	-	12 (39)	-	16 (59)
	4	-	1 (3)	-	1 (4)
		mean (SD)	mean (SD)	mean (SD)	mean (SD)
		0.193 (0.4)	2.452 (0.6)	0.190 (0.4)	2.667 (0.6)
	Score	n (%)	n (%)	n (%)	n (%)
PI	0	20 (65)	8 (26)	14 (67)	5 (19)
	1	10 (32)	4 (13)	6 (29)	9 (33)
	2	1 (3)	19 (61)	1 (4)	11 (41)
	3	-	-	-	2 (7)
		mean (SD)	mean (SD)	mean (SD)	mean (SD)
		0.387 (0.6)	1.355 (0.9)	0.381 (0.6)	1.385 (0.9)
	mm	n (%)	n*(%)	n (%)	n (%)
PD	1	-	-	4 (19)	-
	2	3 (10)	5 (17)	13 (62)	9 (33.3)
	3	21 (68)	13 (43)	4 (19)	9 (33.3)
	4	5 (16)	11 (37)	-	9 (33.3)
	5	2 (6)	1 (3)	-	-
		mean (SD)	mean (SD)	mean (SD)	mean (SD)
		3.193 (0.7)	3.27 (0.8)	2.00 (0.6)	3 (0.8)
		n (%)	n (%)	n (%)	n (%)
BOP		21 (67)	29 (94)	3 (14)	23 (85)
GCF		mean (SD)	mean (SD)	mean (SD)	mean (SD)
µl		0.156 (0.1)	0.335 (0.3)	0.111 (0.1)	0.276 (0.2)

* one PD value missing

analysis, using univariate paired t-tests, demonstrated significantly higher levels for all six proteins (α2-M, α1-AT, TF, Alb: $p<0.001$; IgG: $p=0.036$; LF: $p=0.021$) in GCF from inflamed compared to healthy tooth sites (Table 26). Similarly, repeated measures MANOVA demonstrated a significant effect when the absolute amounts of the six proteins in PISF from healthy and inflamed sites were compared ($p=0.004$). Univariate paired t-tests, comparing the levels of these proteins between healthy and inflamed implant sites, demonstrated that the levels of α2-M, α1-AT, TF, Alb (ng/30s) and IgG (EU/30s) were significantly higher ($p<0.01$) in PISF from inflamed sites (Table 26). However, although LF levels showed a tendency towards increased levels in PISF from inflamed sites, this increase failed to reach statistical significance ($p=0.097$) (Table 26). When the absolute amounts of each protein in fluid from around healthy implant and tooth sites were compared, no significant differences were noted (MANOVA, $p=0.299$). Similarly, when absolute amounts from inflamed tooth and implant sites were compared, the MANOVA did not demonstrate a significant effect ($p=0.06$) (Table 26).

When results were expressed as ng/μg Alb (α2-M, α1-AT, TF and LF) or EU/μg Alb (IgG) the picture was divergent (Table 27). Repeated measures MANOVA demonstrated a significant effect when inflamed and healthy tooth sites ($p=0.011$) were compared. However, univariate analysis demonstrated that the only significant differences were the reduction in IgG and TF levels in GCF from diseased sites

Table 26: Absolute amounts of α2-M, α1-AT, TF, LF, Alb (ng/30s) and IgG against *P.gingivalis* (EU/30s) in GCF and PISF from healthy and inflamed implants and teeth. Geometric means and 95% confidence intervals are shown in parentheses.

	Implant sites		Tooth sites	
	Healthy n=31	**Inflamed n=31**	**Healthy n=21**	**Inflamed n=27**
α2-M	19 (12-30)	49 (28-83)	13 (9-18)	50 (34-73)
α1-AT	36 (25-53)	108 (77-151)	38 (28-50)	136 (99-187)
TF	37 (25-54)	89 (64-125)	41 (29-57)	97 (69-137)
LF	19 (11-35)	32 (19-54)	32 (19-55)	60 (31-116)
IgG	90 (72-104)	125 (101-155)	82 (74-91)	136 (105-175)
Alb	1086 (661-1786)	2360 (1549-4467)	711 (436-1161)	2685 (1660-4355)

MANOVA healthy v inflamed teeth, n=17 pairs, p=0.021; all univariate comparisons significant p<0.05
MANOVA healthy v inflamed implants, n=31 pairs, p=0.004; all univariate comparisons significant p<0.05 except for LF p=0.097
MANOVA healthy implants v teeth, n=21 pairs, p=0.299
MANOVA inflamed implants v teeth, n=27 pairs, p=0.06
v=versus

(p=0.001 and p=0.024 respectively, Table 27). GCF α2-M, α1-AT and LF levels did not demonstrate a significant difference between healthy and inflamed tooth sites when expressed as ng/μg Alb. Similarly, MANOVA demonstrated a significant effect when PISF levels of these proteins from healthy and diseased implant sites were compared (p=0.007), but univariate analysis demonstrated a significant difference only in the reduction of the levels of IgG in PISF from inflamed compared to healthy implant sites (p=0.03). Finally, MANOVA did not demonstrate a significant effect when healthy or inflamed tooth and implant sites were compared (p=0.193 and p=0.137 respectively).

Table 27: Specific amounts of α2-M, α1-AT, TF, LF (ng/μg Alb) and IgG against *P.gingivalis* (EU/μg Alb) in GCF and PISF from healthy and inflamed implants and teeth. Geometric means and 95% confidence intervals are shown.

	Implant sites		Tooth sites	
	Healthy n=31	**Inflamed n=31**	**Healthy n=21**	**Inflamed n=27**
α2-M	17 (10-28)	20 (12-32)	17 (10-30)	19 (11-30)
α1-AT	33 (25-45)	42 (30-60)	52 (35-76)	51 (36-72)
TF	34 (25-46)	35 (24-51)	56 (39-79)	36 (27-48)
LF	17 (10-30)	14 (8-23)	44 (22-87)	23 (12-41)
IgG	82 (53-126)	47 (31-71)	115 (72-182)	50 (32-78)

MANOVA healthy v inflamed teeth, n=17 pairs, p=0.011; significant univariate comparisons TF:p<0.05 and IgG:p<0.01
MANOVA healthy v inflamed implants, n=31 pairs, p=0.007; singificant univariate comparisons IgG: p<0.05
MANOVA healthy implants v teeth, n=21 pairs, p=0.193
MANOVA inflamed implants v teeth, n=27 pairs, p=0.137
v=versus

3.2.3. Cross-sectional study of local antibody levels

The median titre of IgG antibodies against *P. gingivalis* and median concentrations of stromelysin and TIMP are given in Table 28. A MANOVA was performed on these results as for section 3.2.2. and the p-value is given for the within-subjects or treatment effect. Table 29 shows the resultant paired t-tests for this analysis comparing the three disease states. Stromelysin and TIMP show a similar pattern to the acute-phase proteins in being unable to distinguish gingivitis and periodontitis. Specific antibody levels, however, show a tendency towards a distinction between these two states, which is echoed in the greater difference between health and gingivitis than between health and periodontitis. A parallel can also be seen between these findings and those of section 3.2.1. in which deeper pocket depths yielded lower antibody levels.

Table 28: Median titre (EU) of IgG antibodies against *P. gingivalis* and median concentration of stromelysin and TIMP (ng/ml) in GCF from healthy, gingivitis and periodontitis sites.

	Healthy	Gingivitis	Periodontitis	p-value (within subjects)
IgG against *P. gingivalis*	13 (8-19)	39 (10-76)	16 (8-57)	0.006
Stromelysin	8.3 (0-15.2)	14.9 (0-24.1)	18.9 (9.7-25.2)	0.011
TIMP	44.3 (20.4-67.1)	93.6 (35.1-147.4)	85.6 (57.3-166.7)	0.007

Table 29: Paired t-test results for comparisons between healthy, gingivitis and periodontitis sites.

	Healthy/ Gingivitis	Healthy/ Periodontitis	Gingivitis/ Periodontitis
IgG against *P. gingivalis*	0.008	0.034	0.187
Stromelysin	0.058	0.001	0.397
TIMP	0.055	0.002	0.753

3.2.4. Cross-sectional comparison of natural teeth and implants

Table 30 shows the median titres of antibodies to *P. gingivalis* in GCF around natural teeth as compared with PISF around implants. There is a tendency towards higher IgG levels in natural teeth. This is also observed for IgG against *A. actinomycetemcomitans* in Table 31.

Table 30: Median titre (EU) of antibodies against *P. gingivalis* in GCF around teeth and PISF around implants.

	Teeth	Implants	p-value
IgG	22 (20-49)	20 (17-25)	0.06
IgM	17 (15-19)	16 (15-18)	0.22
IgA	26 (21-30)	28 (24-31)	0.45

p-value given for t-test using null-hypothesis (log transformed data).

Table 31: Median titre (EU) of antibodies against *A. actinomycetemcomitans* in GCF around teeth and PISF around implants.

	Teeth	Implants	p-value
IgG	27 (21-30)	23 (20-30)	0.10
IgM	29 (29-34)	30 (29-35)	0.56
IgA	29 (27-35)	30 (27-39)	0.15

p-value given for t-test using null-hypothesis (log transformed data).

3.2.5. Potential of RCE to distinguish healthy and periodontitis sites

Table 32 shows the RCE values for each antibody class for both periodontitis and healthy sites. The reliability of the RCE ratio in the evaluation of the disease status of a site is summarized in Table 33.

Table 32: RCE values for IgG, IgA and IgM from periodontitis and healthy sites.

	Periodontitis	Healthy
IgG	2.40(0.62-2.78) 80%	1.44(0.00-18.15) 40%
IgA	2.09(1.17-2.44) 100%	0.81(0.09-3.35) 60%
IgM	0.20(0.03-1.64) 20%	0.38(0.00-2.60) 70%

Results reported as RCE (range) and % of periodontitis observations with RCE>1 or healthy observations with RCE<1.

Table 33: Specificity, sensitivity and accuracy of RCE values in evaluating disease state.

	Specificity	Sensitivity	Accuracy
IgG	75%	50%	58%
IgA	100%	62%	75%
IgM	55%	33%	50%

Chapter 4

Discussion

4.1. Systemic Response

4.1.1. Experimental gingivitis study

The results presented here on changes in systemic antibody titres to three microorganisms which were found to be predominant in the subjects studied, do not show any significant differences over a 28-day period. This is in accord with previous findings that serum antibody responses to Gram-positive organisms do not yield useful data in relation to periodontal disease (135-138). It would also perhaps have been surprising if any such systemic effects had been detectable after such a short period of experimentally-induced gingivitis.

In this early study, the ELISA technology had not yet been modified sufficiently to have the sensitivity to quantify local antibody levels. However, the acute-phase protein, α2-macroglobulin (α2-M) and the iron-binding protein, transferrin (TF) were assayed in GCF to monitor the progression of inflammation.

α2-macroglobulin (α2-M) is a plasma protease inhibitor (MW 725kd) (253). Other work in this laboratory indicates that α2-M is normally present in gingival crevicular fluid (GCF) at about 10% of the serum level (2.2-3.8 mg/ml). It is important in the inhibition and ultimate removal of potentially harmful endoproteases, either exogenous or endogenous (254). The protective function of α2-M is not

confined to the blood circulation as it is also found in other body fluids including gingival crevicular fluid (GCF). In a recent study it was found that the total GCF concentration of α2-M correlates inversely with the proportion of alveolar bone loss in patients with severe periodontitis (255). These authors also reported that α2-M total concentration was lower in GCF collected from sites with more inflamed gingiva in patients with gingivitis.

Transferrin is a serum-derived glycoprotein with a molecular weight of 81kd (256). It functions in serum as an iron transport protein between sites of absorption, utilisation, storage and excretion (257). Its serum level is normally around 3mg/ml (258), although this can be raised in various inflammatory conditions. In GCF it could function as an antibacterial agent, in conjunction with lactoferrin and other iron-binding proteins, to produce an iron-limiting micro-environment (259). Further, transferrin has a role in the cell-mediated immune response and is required for T-cell transformation and macrophage activation (260). Transferrin was first identified in GCF by Schenkein and Genco (261) in levels similar to those of α2-M.

Figure 1 shows the changes in α2-M and TF concentration in GCF during the study. α2-M increases sharply at an early stage in inflammation and then falls off, recovering at the end of the study period. This suggests that α2-M levels increase as a response to inflammation (probably augmented by local production) but

decrease as α2-M is consumed by greatly increased levels of host and bacterial proteases. By contrast, TF levels fall off consistently throughout the study period, reflecting the purely serum-derived nature of this protein.

In addition, Table 2 demonstrates the ability of *P. gingivalis* to degrade both of these proteins to a much greater extent than that evidenced by the three Gram-positive organisms studied. In the light of these preliminary findings, it was decided to concentrate further studies of the humoral immune response on *P. gingivalis*.

4.1.2. Study of antibody avidity related to attachment loss

The relationship between antibodies to various suspected periodontopathogens and periodontal status has been extensively investigated although many of the findings have been contradictory and this remains a highly controversial field. Ebersole and Holt (262) have dealt with the application of antibody titres to diagnostics in great detail. However, Wilton *et al* (134), in their review, stated that the data currently available do not allow these responses to be diagnostic. Generally, reports in this field are agreed that an elevation in the humoral immune response to certain plaque-associated microorganisms is detected in the presence of and/or increasing extent of periodontitis (134, 146). However, a few studies have suggested the opposite tendency (140, 154).

Antibody avidity, that is the measure of the net

binding strength of antibody to antigen, has been extensively studied in a number of fields, both in relation to antibody titre and in isolation, in terms of disease susceptibility and progression. There have, however, been very few reports dealing with antibody avidity in relation to periodontal disease, as reviewed in the introduction. The investigation of Ebersole *et al* (200) studying the increase in avidity in the non-human primate, *Macaca fascicularis*, following immunization with tetanus toxoid, previously discussed, found that IgG avidity increased from 0.9M to 1.72M following primary immunization and 2.56M after secondary immunization. Lopatin *et al* (201) demonstrated that avidity of antibody rose to a similarly high level in rabbits post-immunization with *P. gingivalis*, but that human antibodies to this organism appear to be of generally low avidity. This latter finding was confirmed by the present study. The present report also confirms the finding of Lopatin *et al*, and others (148, 214, 263), that IgG serum antibody titres to *P. gingivalis* are significantly higher in periodontitis patients on maintenance than in controls ($p<0.01$) (Table 3 and Figure 2). Additionally it is shown that this is also the case for IgA antibodies, but not IgM. However, it was not possible to demonstrate a statistically significant difference in antibody avidity for any immunoglobulin class in the above subject groups. The observation that IgM avidity to *P. gingivalis* is higher in NAL than in AL patients is difficult to explain,

although perhaps the metabolic block referred to by Panoskaltsis and Sinclair (199) preventing IgM-IgG switching and leading to increased IgM avidity is operating here. Nevertheless, the finding may have prognostic significance if confirmed by further studies.

Chen *et al* (157) concluded that after periodontal treatment of rapidly progressive periodontitis patients, their IgG antibody avidity increased. The data from the present study presented in Table 5 may reflect the treatment effect reported by these authors. In this case the treatment effect would generate a strong and statistically significant positive correlation between titres and avidities of IgG antibodies to *P. gingivalis* in NAL patients as seen in Table 7. This would result from increased avidities and decreased titres stimulated by treatment, or in other words, a shift to more biologically effective antibodies. However, the present study dealt with adult periodontitis patients in maintenance phase, and Chen *et al* examined rapidly progressive periodontitis patients prior to, and after initial treatment, therefore the two studies are not strictly comparable.

Another recent study of titre and avidity of IgG antibodies to *P. gingivalis* in rapidly progressive periodontitis by Whitney *et al* (202) also shows lower avidities in RPP patients than in controls. This contrasts with the present study which demonstrated a trend towards higher IgG avidities in adult periodontitis patients than in controls ($p=0.065$). Moreover, if seropositive control

subjects are excluded using the criterion of Chen *et al* (157), i.e. titre > 2x median control titre, then this yields a significant difference between seronegative control subjects and adult periodontitis patients (p=0.02). These contrasting findings for these different periodontitis patient groups may reflect a differing aetiology in terms of immunological susceptibility.

The longitudinal aspect of this study failed to show any significant changes in titre or avidity of antibodies to *P. gingivalis* during the three month monitoring period (Table 5). The non-statistically significant increase of 0.46M in AL patients compared with a decrease of 0.10M for NAL patients (p=0.14) raises speculation about long-term changes in antibody avidity which can only be answered by a further longitudinal study over a longer time period.

The comparison of the responses to *P. gingivalis* and *A. actinomycetemcomitans* is interesting in that the avidity of IgG antibodies to the latter was found to be significantly lower than to the former (Table 6). However, only the periodontitis group were seen to have a significant divergence in response; i.e. equivalent to 6-7 times the binding strength, using the scale of Ebersole et al (200). The relevance of this is unclear but there is a suggestion that not only do IgG avidities to *P. gingivalis* tend to be higher in periodontitis, but IgG avidities to *A. actinomycetemcomitans* tend to be lower than normal as shown in Table 6. This may indicate the differing relevance of these two organisms in adult

periodontitis.

A very recent study by Sjostrom *et al* (203) showed that IgG antibodies in low-titre sera from controls subjects were significantly more effective in opsonizing *A. actinomycetemcomitans* than IgG antibodies in low-titre sera from RPP patients. The finding of the present study that avidities of IgG antibodies to *A. actinomycetem-comitans* tend to be lower in adult periodontitis patients than in control subjects, although not statistically significant, may indicate a failure of biological function of antibody directed against *A. actinomycetemcomitans* in these patients also.

Unlike Lopatin *et al* (201), the present study has demonstrated a significant correlation between titre and avidity of the IgG response to both organisms studied. In addition, these data show that there are differences between patient groups in relation to these correlations. This indicates the potential prognostic value of antibody avidity in determining patients susceptibility to further attachment loss.

4.1.3. Comparison of antibody avidity in various forms of periodontal disease

The present study shows that IgG and IgM antibody avidities to *P. gingivalis* were lower in RPP patients than AP patients, with IgA titres being significantly lower than in AP, and tended to be lower than in age/sex matched controls. However, a contrasting pattern could be discerned for *A. actinomycetemcomitans*, with IgA avidity tending to be higher in RPP than in AP and IgG and IgA titres also being significantly higher. As such this study supports the hypothesis that there are differences in the humoral immune response to these two organisms in RPP and AP, and is consistent with previous studies demonstrating that avidities to *P. gingivalis* are lower than normal in RPP (157, 202) and higher than normal in AP (201) (Tables 8-11, Figures 7-10). In relation to AP avidities, this also confirms the findings of the previous Section (4.1.2.).

RPP has been recognized as a distinct clinical condition (264, 265). In addition, an association has been demonstrated between the occurrence of RPP and the HLA system (266, 267). A correlation has been demonstrated between HLA A9 and RPP. A number of studies have been published showing that RPP patients differ in their humoral immune response expressed as antibody titre to suspected periodontopathogens (268-271).

A recent study by Ebersole and Kornman (206)

demonstrated that *P. gingivalis* emerges as an organism in the subgingival plaque during the conversion from gingivitis to progressing periodontitis in a non-human primate model, and that this elicits a systemic antibody response specific for this microorganism. Similarly, a study by Dahlen and Slots (18) in rabbits showed that animals co-inoculated with *P. gingivalis* and *A. actinomycetemcomitans* showed significantly more severe disease than animals which were monoinfected. They conclude that the immune system acting through systemic antibodies and/or cellular mechanisms may modulate the pathogenic potential of infecting periodontal pathogens. It may be that antibody avidity has a crucial role in this modulation.

In the light of these findings and evidence of the importance of avidity as an aspect of the biological function of antibody (204, 205, 272), it may be that RPP patients constitute a sub-group which is predisposed to early and rapidly progressive disease as a result of a mechanism blocking the production and/or deployment of strongly-binding antibody to suspected periodontopathogens.

The findings presented here of overall low-avidity antibodies in RPP patients, and generally in humans, are in agreement with previous reports (157, 201, 202). They are also in accord with the results of the previous Section (4.1.2.). However, they contrast with higher avidities of antibody to *P. gingivalis* and *A. actinomycetemcomitans* found in an early study in this field (168). It must be borne in mind, however, that these data were based on

samples of five and four patients respectively in a mixed periodontitis group, and that these samples may have contained an unrepresentatively high proportion of seropositive AP patients. As the present study shows, these, and a small percentage of seropositive RPP patients can skew analyses unless measures are taken such as dichotomization into seropositive and seronegative groups or non-parametric testing based on medians rather than means.

The data presented in Table 15 on the relationship between titre and avidity are in general agreement with the findings of the previous Section (4.1.2.). A clear difference can be discerned between periodontitis patients and control subjects, and there is also an indication that these relationships in terms of the responses to *P. gingivalis* and *A. actinomycetemcomitans* may also yield a useful distinction between AP and RPP patients. It is interesting to note that all of the correlations here were positive compared with the negative correlations between titre and avidity of antibody to *Candida albicans* found in a recent study (273).

The data presented here demonstrating the ability of a discriminant analysis to successfully classify RPP patients based on antibody avidity also shows that misclassification of AP patients also occurs. This requires a prospective study employing other parameters, e.g. radiographic evidence, to establish the value of antibody avidity to *P. gingivalis* as an aid in the

differential diagnosis of AP and RPP.

4.1.4. Investigation of treatment effect on systemic antibody

An early study by Ebersole's group (135) investigated the ability of serum antibody titres to distinguish periodontitis from healthy subjects and suggested that these responses could provide an appraisal of treatment effects in the management of periodontitis patients. Naito *et al* (148) demonstrated that the serum titre to *P. gingivalis* was reduced in periodontitis patients post-therapy. This has been confirmed by Aukhil *et al* (173). The treatment regime employed here included scaling, root planing and oral hygiene instruction, followed by surgery or scaling and root planing under anaesthesia.

However, Mouton *et al* (212) reported that a reduction of 50% in antibody titres to *P. gingivalis* occurred within one year post-treatment in most patients, after an initial increase. A sub-group of patients, however, did not respond to the challenge of the mechanical treatment regimen by producing an increased antibody titre to *P. gingivalis*.

An investigation of antibody titres to *A. actinomycetemcomitans* in LJP (274) has shown that these decreased post-treatment. It has been shown that untreated AP patients have significantly elevated antibody titres to *P. gingivalis* compared with treated AP patients (275).

Additionally, an investigation of serum titres to *P. gingivalis* LPS in relation to treatment effects demonstrated levels reduced by 15-30% long-term post-treatment (276). Ebersole *et al* have reported that the homologous organism can be detected in subgingival plaque when antibody titre elevations are found post-scaling (277), and also that multiple foci of infection must be successfully treated to have an effect on serum IgG levels (278). They have also demonstrated that the long-term reductions in antibody titres occur most frequently 8-10 months post-therapy, within a range of 2 months- 2 years (262).

A recent report by Ebersole *et al* (279) quotes data showing that 60% of periodontitis patients exhibited increased titres to a battery of suspected periodontopathogens post-treatment, and that these patients had fewer episodes of active disease during the monitoring interval (two years) than those who did not demonstrate increased titres.

The available literature on this subject concurs that increases in antibody titre to *P. gingivalis* occur post-therapy in most periodontitis patients and thus could have a bearing on treatment outcome. In addition, long-term reductions in antibody level are associated with the resolution of infection.

However, these studies have focused on antibody titre and have not considered antibody avidity in relation to treatment effects. Chen *et al* (157) studied both titre and

avidity of IgG antibodies to *P. gingivalis* before and after treatment in RPP patients. They found that, although avidities are significantly lower in RPP patients pre-treatment than in controls, these avidities increased significantly post-treatment to levels higher than in controls. They concluded that many RPP patients do not produce protective levels of biologically functional antibody during the course of natural infection, but that they may be stimulated to do so by treatment.

These conclusions are borne out by the findings of the present study (extended to the investigation of IgG, IgM and IgA avidity and titre against *P. gingivalis* and *A. actinomycetemcomitans*) in relation to IgG avidity to *P. gingivalis* (Table 16 and Fig.11), IgA titre to *P. gingivalis* (Table 17 and Fig.12), and IgG,IgM and IgA titre to *A. actinomycetemcomitans* (Table 19 and Fig.14).

The effect of treatment on antibody responsiveness may result from an inoculation effect during scaling and root planing, or from the reduction in antigen load resulting from these procedures and improved oral hygiene, or from a combination of these two mechanisms. Reduction in antigen load is known to result in selection of B-cell clones producing higher avidity antibodies (2). Treatment may also permit the development of a normal maturation of the immune response, perhaps mediated by an inoculation effect, leading to antibodies of increasingly higher avidity.

Both the present study and that of Chen *et al* (157)

demonstrate that IgG avidities to *P. gingivalis* are subject to a general increase in all patients post-treatment, the present study dealing with AP patients and Chen *et al*'s study with RPP patients. However, when patients are dichotomized into seropositive and seronegative groups, using Chen *et al*'s criterion of titre > 2x control median titre, then both studies show convincing increases in IgG titre to *P. gingivalis* only in the seronegative group. The present study found that IgG titre increased in 10/11 (91%) of seronegative patients, but only 3/8 (38%) of seropositive patients. Chen *et al* suggest that some patients (seropositive) are producing large amounts of low-avidity, biologically ineffective antibody until therapy induces production of higher-avidity antibodies required at lower concentration; whereas others (seronegative) are producing very little specific antibody until therapy induces production of higher-avidity antibodies at much higher concentration.

4.2. Local Response

4.2.1. Comparison of local antibody titres in adult periodontitis patients (sites with differing clinical indices)

In the present study specific IgG levels against *P. gingivalis* were found to be significantly lower in more

severe chronic periodontitis sites as defined by PD and GI. However, no significant differences in IgA levels were found between chronic periodontitis sites of varying disease levels. Baranowska *et al* (207) found no difference between levels of specific IgG to *P. gingivalis* in healthy and diseased sites within the same individual. However, fixed volume samples of 0.5µl were taken. This means that strips from sites which did not yield 0.5µl were discarded and that samples were taken over variable time periods resulting in variable rates of serum contamination (280). Since healthy sites tend to yield lower volumes than diseased sites, this method treats healthy and diseased sites in different ways. Therefore, these results are not strictly comparable with the present study. An earlier study by Schenck (211) showed an inverse relationship between the number of deep pockets (>4mm) and serum antibody level to *P. gingivalis* lipopolysaccharide in chronic periodontitis patients. Mouton *et al* (212) demonstrated a dichotomy in serological responses to *P. gingivalis* among chronic periodontitis patients with one sub-group exhibiting high serum antibody levels and another having levels similar to those of healthy individuals. The patients with high serum antibody levels appeared to respond better to therapy as evidenced by reduction in the number of deep pockets within one month post-treatment. Their findings are consistent with the data reported here that lower *P. gingivalis* IgG titres are found in more severely affected sites.

A fairly strong correlation between IgG, but not IgA, to *P. gingivalis* in serum and gingival crevicular fluid was found in this study (Table 21) and similar results have been reported previously (207). However, other studies have reported lower mean gingival crevicular fluid than serum IgG levels to *P. gingivalis* (281) as well as lower total IgG in gingival crevicular fluid than in serum (282). In addition, Lamster *et al* (210) reported a significant correlation between total IgG in gingival crevicular fluid and specific serum antibody to *Bacteroides intermedius* (now *Prevotella intermedia*) but not *P. gingivalis*. They conclude that the development of a serum immunoglobulin response to suspected periodontopathogens is consistent with a protective host response. A corollary of this view is that a local deficiency of IgG to *P. gingivalis* may lead to local disease progression. Alternatively, Kilian (21) has demonstrated that *P. gingivalis* can degrade human IgG and IgA, suggesting that low gingival crevicular fluid levels of IgG may be caused by degradation by *P. gingivalis*, or that locally available antibodies are adsorbed by the greater mass of subgingival plaque present.

Levels of serum and gingival crevicular fluid IgG against *A. actinomycetemcomitans* were found to be highly correlated in this study. In contrast to the results presented on *P. gingivalis* IgG, there were no differences in gingival crevicular fluid IgG levels to *A. actinomycetemcomitans* in chronic periodontitis sites of varying disease levels (Table 20 and Fig.15). Gunsolley *et al*

(216) found that serum antibody levels to *A. actinomycetemcomitans* and *P. gingivalis* are inversely related to the degree of periodontal destruction in young adults with juvenile periodontitis or generalized severe periodontitis and concluded that this is consistent with a hypothesis that failure to mount a substantial antibody response to these organisms leads to more widespread periodontal destruction. The present study of adult periodontitis patients would support this view at both local and systemic levels, but only for *P. gingivalis*.

Genco *et al* (283) showed that adsorption of serum from localized juvenile periodontitis patients with *A. actinomycetemcomitans* and *H. aphrophilus* resulted in the elimination of most of the reactivity to the homologous strain but not to the heterologous strain. In the present study most of the reactivity to *A. actinomycetemcomitans* could be removed by adsorption with both organisms (data not shown) suggesting that there is cross-reaction between antibodies to these two species. Similarly, strong correlations were found between serum IgG and IgM levels to these two organisms. The indications are that antibodies detected to *A. actinomycetemcomitans* in adult periodontitis patients could be confounded with cross-reacting antibodies directed against *H. aphrophilus*, which is, with other Haemophili, commonly isolated from dental plaque (284).

Ebersole *et al* (169) demonstrated in patients with various types of periodontal disease, that the same

organism to which the individual exhibited elevated serum antibody was detected in 55% of disease-active sites but only 18% of disease-inactive sites. However, Williams *et al* (285) in a study of patients with early-onset periodontitis, found that many patients had serum antibodies to organisms not found in the pocket flora and that some organisms which made up a large proportion of the pocket flora did not seem to provoke a corresponding antibody response. These authors suggested that sequential infection occurs in these forms of periodontitis leading to induction of protective immunity against reinfection by the same organism.

In conclusion, there appears to be general agreement that antibody levels to *P. gingivalis* are raised in periodontitis patients as compared with healthy individuals but there are also indications, supported by the present study, that lower antibody levels may be present in the more disease affected sites within the periodontitis group. In addition, this study failed to demonstrate any significant differences between gingival crevicular fluid IgG levels against *A. actinomycetemcomitans* between sites with different pocket depths and gingival indices. Care must be taken, however, in interpreting these results as although these patients exhibited sites with pocketing and gingivitis (Table 20 and Fig.15), they were maintenance regime patients who have received extensive periodontal treatment. Thus they may not be strictly comparable with

non-treated adult periodontitis patients. This study, however, addresses the cross-sectional comparison of healthy and diseased sites and the correlation between local and systemic antibody titres. Further work is required to elucidate the inverse relationship between the clinical indices and gingival crevicular fluid and serum IgG antibody to *P. gingivalis* found in the periodontitis patients of the present study.

4.2.2. Comparison of humoral immunity in GCF and PISF

The long-term success of osseointegrated dental implants relies on the maintenance of the biological seal and the integrity of the peri-implant tissues (286). The peri-implant supracrestal tissues are in many ways comparable to their periodontal counterparts (287). Like the gingival crevice at a tooth, the peri-implant sulcus provides a niche for the establishment and growth of oral microorganisms. The microflora around natural teeth and implants have been compared in partially edentulous patients, and were found to be analogous (288-290). In addition, an animal study has shown that microbial colonization and establishment on titanium osseointegrated implants, from healthy mucosa to experimental mucositis and peri-implantitis, follow a very similar pattern to that of natural teeth (291).

One issue which has recently attracted attention, is whether periodontal (gingiva) and peri-implant mucosa share

the same inflammatory and immunological responses. The inflammatory infiltrate in peri-implant healthy and inflamed mucosal biopsies from humans has been immunohistochemically characterised and resembles that found in the gingival tissue. It comprises mainly lymphocytes, macrophages and only very few plasma cells (292). Moreover, histological studies on animals have demonstrated that the reaction of the soft tissues to early plaque formation is similar around teeth and implants (293). Long term animal studies, however, have shown that prolonged plaque accumulation and inflammation may result in a greater apical extension of the inflammatory infiltrate around implants than teeth (294) and that in experimental peri-implantitis, inflammatory cells may be found even in the adjacent bone marrow (295). Thus, a more intense inflammatory response may develop around implants when compared to teeth in response to prolonged plaque accumulation, although further human studies are needed to confirm this.

Gingival crevicular fluid (GCF) or peri-implant sulcus fluid (PISF) provide a non-invasive means of comparing the inflammatory and immunological responses around natural teeth and implants. However, in contrast to GCF which has been widely examined (259), very few studies exist on PISF components and their relation to peri-implant status (289, 296).

The protease inhibitors, α2-M and α1-AT play an important role in the neutralisation of the proteases

released in the gingival crevice (297), whereas the iron-binding proteins TF and LF may act as antimicrobial agents in the area (259). These acute-phase proteins have been previously identified in GCF (255, 298-302). In addition, it has recently been demonstrated that their absolute amounts are elevated in GCF from 'diseased' compared to 'healthy' sites (246, 247), and also that LF reflects PMN numbers in the crevice (247). Thus, these acute-phase proteins may also serve as markers of peri-implant inflammatory status.

Local GCF immunoglobulin levels against putative periodontal pathogens, including *P. gingivalis*, may provide a measure of the humoral immune response at a specific site. The results of Section 3.2.1. showed that GCF antibody titres against *P.gingivalis* were lower in deep pockets and inflamed sites than in shallow pockets or less inflamed sites. As *P. gingivalis* has been isolated also from the subgingival microflora around implants in partially edentulous patients (288-290), PISF IgG levels against this periodontal pathogen may provide useful information on the immunological status of peri-implant sites.

The clinical data displayed some interesting features. Very few inflamed implant and tooth sites had an MGI score of 4, demonstrating that implants and teeth were relatively well maintained. As expected, inflamed implant and tooth sites demonstrated higher amounts of plaque than healthy sites depicting, within the limits of this cross-sectional

investigation, the well-known association of plaque accumulation with inflammation of the gingival tissues but also of the peri-implant tissues. The allocation of implant and tooth sites to the clinically healthy or inflamed group was done on the basis of the MGI, which assesses only visual changes of the soft tissues and thus, does not interfere with GCF or PISF sampling. When the clinically healthy sites were probed after all sampling was completed, 3 tooth sites (14%) compared to 21 implant sites (67%) bled following the probing. This difference in bleeding frequency is most likely to be due to the reduced resistance to probing offered by the peri-implant mucosa in comparison to that offered by the gingiva, as a consequence of differences in terms of tissue composition, organisation and attachment to the root/implant surface (303). In fact, in a recent beagle dog study (304), in which probing with standardised pressure (0.5N; probe diameter 0.5mm) at implants and teeth with non-inflamed mucosa was histologically evaluated, it was reported that the probe penetrated on the average 1.3mm (SD 0.3) into the supracrestal connective tissue at implants, while at teeth the tip of the probe was consistently located within the zone of the junctional epithelium. The high frequency of bleeding found in the present study following probing of the 'clinically healthy' implant sites, despite the use of a probing pressure of only 0.25N, indicates that a pressure less than 0.25N may be needed in order not to traverse the apical termination of the peri-implant junctional

epithelium, and hence caution must be exercised when comparing the probing data for teeth and implants in the present investigation.

The assessment of the four acute-phase proteins, Alb and also IgG against *P.gingivalis* provides a profile for GCF and PISF components of different sources and with different functions. Such a profile, especially when components are assessed in GCF or PISF from the same site, gives a picture of the inflammatory and immunological responses at a specific site. None of the constituents reported in this study have been previously determined in PISF. Similarly to GCF, PISF demonstrates protease-inhibitory capacity due to the presence of the potent protease-inhibitors α2-M and α1-AT, as well as general antimicrobial properties via the iron-binding proteins TF and LF. The presence in PISF of specific immunoglobulin against *P.gingivalis*, a putative periodontopathogen that has been isolated from peri-implant sulci (288-290), could be due to localised induction of plasma cells of the peri-implant mucosa to IgG production and/or leakage from serum. Specific immunoglobulin in the peri-implant sulcus could be involved in the opsonisation of micro-organisms and also complement activation rendering further specific antimicrobial properties to PISF.

In this investigation, it was decided to express results of GCF and PISF constituents in two ways, as ng/30s sample and as ng/μg Alb. The rationale for expressing results as ng/30s sample has been discussed previously

(244). Reporting results per unit Alb provides information on constituent levels relative to the serum derived component of GCF or PISF and may thus supply an indication of proteins derived from the systemic circulation.

It has been shown in previous investigations that the levels of the acute-phase proteins are higher in GCF from inflamed tooth sites compared to healthy sites (246, 299, 302). Thus, when the results were expressed as ng/30s, the increase at inflamed sites was anticipated, for the protease inhibitors α2-M and α1-AT and the iron-binding proteins TF and LF (Table 26). For α2-M, α1-AT and TF, which have high serum concentrations (2mg/ml) (256), increased vascularity and vascular permeability is probably the major factor contributing to higher GCF or PISF levels at inflamed sites. This is supported by the simultaneous rise in GCF or PISF volume as well as Alb at inflamed sites around both implants and natural teeth. Further support for the predominantly serum origin of α2-M and α1-AT is provided by the inability to demonstrate a significant difference between healthy and inflamed sites for both implants and teeth when the results were expressed relative to Alb (Table 27).

When results were expressed as ng/30s LF demonstrated a statistically significant increase in GCF from inflamed compared to healthy tooth sites. As LF may be considered a crevicular PMN marker (247) its increase in GCF at inflamed sites may be accounted for by increased PMN emigration. Although a significant difference was not

shown when inflamed implant and tooth sites were compared, there was a tendency for inflamed implant sites to have lower levels of LF than inflamed tooth sites. This tendency is in line with findings reported from a recent animal study showing that lower PMN numbers may be found in inflamed peri-implant tissue compared to inflamed tooth sites (294).

The finding that inflamed tooth sites demonstrated higher antibody titres (EU/30s) of IgG against *P.gingivalis* than healthy tooth sites appears to contradict the findings of Section 3.2.1. However, one has to consider that the present study refers to non-periodontally involved sites from well-maintained patients, whereas the previous study was on a periodontitis affected population. In addition, Table 27 demonstrates that specific IgG titres to *P.gingivalis* were significantly lower in both GCF and PISF from inflamed sites when expressed as EU/μg albumin. This suggests that in inflamed sites proportionately less specific antibody is derived from serum and proportionately more from the local plasma cell infiltrate. There also appears to be a trend towards IgG titres in PISF from healthy implant sites being lower than in GCF from healthy tooth sites measured on this basis, suggesting the possibility of a difference in the plasma cell infiltrate around implants and natural teeth. This will be explored further in the later discussion of the second implant study.

An interesting observation was the drop in TF and IgG

levels and the tendency for lower LF levels at inflamed tooth sites compared to healthy sites when results were expressed as ng/μg Alb (Table 27). The drop in specific IgG levels in inflamed tooth sites may be related to the opsonisation and/or degradation by *P.gingivalis* resulting in consumption of the immunoglobulin (21). Furthermore, both TF and LF may bind to oral isolates, which may contribute to decreased levels of the iron-binding proteins at diseased sites (305). These events would result in a relative dilution of TF, LF and IgG compared to Alb. At diseased implant sites, however, only IgG showed a trend for lower levels when the results were expressed as ng/μg Alb, which may be related to the tendency for higher Alb levels (ng/30s) at healthy implants sites compared to healthy tooth sites. The tendency for higher albumin levels may suggest that structural and anatomical differences in implant sites result in greater leakiness in healthy implant compared to healthy tooth sites. However, the fact that overall no significant differences were observed between implants and teeth (ng/30s or ng/μg Alb; Tables 26 and 27) suggests that production of PISF is probably governed by similar mechanisms to that of GCF and that inflammatory events are similar in the peri-implant mucosa and the gingiva.

In conclusion, this investigation suggests that great similarities exist in the profile of GCF and PISF constituents and analogous mechanisms seem to control

inflammatory and immunological responses around both implants and natural teeth. However, there is also a suggestion that implants and natural teeth may differ in the local plasma cell infiltrate, and this will be discussed further later.

4.2.3. Cross-sectional study of local antibody levels

A further cross-sectional study of specific IgG titres to *P.gingivalis* was conducted. This differed from the previous study in that three sites were sampled in each patient; one healthy, one gingivitis and one periodontitis site. In addition, an assessment of the levels of the tissue metalloproteinase, stromelysin, and the tissue inhibitor of metalloproteinases (TIMP) was made.

Members of the metalloproteinase family, such as collagenase, stromelysin and gelatinase, are thought to play a role in the natural processes occurring during tissue remodelling and repair. The precise role of these enzymes in the pathological destruction of tissue observed in disease processes such as the arthritides (306, 307), periodontal disease (308) and tumour metastasis (309) is the subject of much current research. It is thought likely that these enzymes are controlled *in vivo* by the rate of activation (310, 311) and through the action of TIMP, which forms tight non-covalent complexes with active forms (312-314).

Most connective tissue cells, including fibroblasts,

chondrocytes and endothelial cells, produce low or undetectable levels of metalloproteinases in monolayer culture. They can, however, be stimulated to synthesise relatively high levels of these enzymes in the presence of mononuclear cell cytokines, e.g. IL-1 (315) and TNF (316). In human gingival or synovial fibroblasts three separate metalloproteinases, collagenase, stromelysin and gelatinase, can be shown to be coordinately induced after stimulation with a variety of cytokines and growth factors (317). These cell types also produce TIMP (318) and several, but not all, of the factors which stimulate enzyme production also stimulate synthesis of the inhibitor (319, 320). These enzymes and TIMP are not present in appreciable concentration in serum in systemic health (313) and, therefore, their presence in GCF can be used as an indicator of local activation and inhibition of tissue destruction.

Table 28 shows that all three parameters, i.e. IgG against P.gingivalis, stromelysin and TIMP show significant p-values for the within-subjects effect (i.e. that the presence of disease within a subject has an effect on the measured level of the parameter).

However, as Table 29 demonstrates, a significant difference between health and gingivitis, as opposed to health and periodontitis, is observed for IgG against *P.gingivalis* only. In addition, there is a definite trend towards specific IgG levels being lower in periodontitis sites than in gingivitis sites. This bears out the

findings of the previous cross-sectional study and suggests that measurement of specific IgG levels in GCF may provide a more sensitive indicator of local disease progression than metalloproteinase or TIMP levels.

4.2.4. Cross-sectional comparison of natural teeth and implants

The further comparative study of GCF from natural teeth and PISF from matched implants was extended in its scope to include all three main immunoglobulin classes, IgG,IgM and IgA, directed against both *P.gingivalis* and *A. actinomycetemcomitans*. The findings are presented in Tables 30 and 31. These accord generally with the findings of the earlier implant study presented in Tables 26 and 27.

This later study demonstrates that there is a tendency towards IgG titres to *P. gingivalis* being higher in natural teeth than in matched implants. This also applies for IgG to *A. actinomycetemcomitans*, although there is an opposite tendency for IgA against *A. actinomycetemcomitans*. This agrees with the data presented for the earlier study in Table 27, which show that IgG titres to *P.gingivalis* are significantly higher in GCF from natural teeth than in PISF from implants when expressed as EU/ug albumin. Statistical significance may also have been achieved in this later study if albumin data had been available.

The other striking point when one comes to compare these two implant studies is the fact that IgG titres were

found to be markedly higher in the earlier than in the later study. Possible explanations for this are the differing periods post-implantation (1-10 years and 6 months-1 year respectively), and the more stringent inclusion criteria in the later study (probing depths < 4mm, absence of visible plaque i.e. PI = 0).

4.2.5. Potential of RCE to distinguish healthy and periodontitis sites

A pilot study was conducted in which total IgG, IgM and IgA levels and albumin levels were assayed in serum and GCF from periodontitis patients and healthy controls. The aim was to apply the RCE ratio, using the method of Giannopoulou *et al* (249) and Out *et al* (250), to assess the extent of local immunoglobulin production.

The data presented in Tables 32 and 33 show that, although there are definite tendencies toward increased local IgG and IgA production and decreased IgM production in periodontitis, the small sample numbers probably had a large influence on the low accuracies achieved.

Immunocytochemical techniques have demonstrated that B-cells and plasma cells are the predominant cell types in periodontitis, producing IgG,IgA and, more rarely, IgM (321). The present study suggests that local production of IgM occurs in healthy sites, but that infiltration of

IgG- and IgA-producing clones may occur in periodontitis. It has recently been suggested that the numbers and proportions of plasma cells producing IgG subclasses might be associated with the stage of periodontitis (322).

These preliminary findings require to be thoroughly investigated using both ELISA technology and immunolocalization techniques. Since conventional immunocytochemistry techniques have been known to produce background-staining problems in this field (323), *in situ* hybridization techniques would appear to provide a better option (324).

These techniques would be especially useful in distinguishing IgG subclass secreting cells, distinguishing secreting plasma cells from memory B-cells on the basis of mRNA copy number (325, 326), and distinguishing IgA1 and IgA2 secreting cells (322, 327).

4.3. Conclusions

The work presented in this thesis has demonstrated that systemic levels of antibody directed against particular suspected periodontopathogens are influenced by periodontal disease type, disease status and the effect of treatment. The differences in avidity of antibody to *P.gingivalis* between AP and RPP patients perhaps point to differences in host related disease susceptibility. Similarly, the differences in antibody avidity between AP patients with different attachment loss experience suggest that the efficacy of the humoral immune response has a bearing on disease progression. The treatment effects seen would suggest that periodontal treatment, such as root planing, provokes an immune response leading to the selection of B-cell clones producing higher affinity antibody.

In terms of site susceptibility, the data presented here suggest that a local deficiency of specific antibody, caused by reduced production or increased absorption and consumption, may lead to local disease progression. There are also indications, based on the findings of two cross-sectional studies, that IgG titres to *P.gingivalis* may allow discrimination between gingivitis and periodontitis sites. This cannot be achieved by markers of inflammation, e.g. acute-phase proteins, or markers of tissue degradation, e.g. matrix metalloproteinases and TIMP.

The comparative studies of implants and natural teeth have suggested that there may be differences in the plasma

cell infiltrate around these fixtures.

All of these areas require much further research. For example, if the rate of immunoglobulin production in the gingival tissue could be determined in periodontitis, gingivitis and health, then an answer may be found to the question of whether lower GCF antibody levels observed in periodontitis sites are attributable to greater local consumption or by a repression of synthesis.

Use of further advanced technologies, e.g. *in situ* hybridization (ISH) and polymerase chain reaction (PCR), will probably be required to elucidate changes in disease stage, different periodontal disease types, and site-specific differences, especially in relation to oral implants. However, the use of antibody avidity assessments also has the potential to further elucidate the immunological basis of periodontal disease susceptibility.

Further studies of the humoral immune response in periodontal disease will require to approach the problem from the various perspectives that these technologies, developed and developing, provide.

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LIST OF PUBLICATIONS

The following publications include material presented as part of this thesis:-

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Kinane DF, Adonogianaki E, Moughal N, Winstanley FP, Mooney J, Thornhill M. Immunocytochemical characterisation of cellular infiltrate, related endothelial changes and determination of GCF acute phase proteins during human experimental gingivitis. J Periodont Res 1991; **26**: 286-288.

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Local and systemic antibody response to putative periodontopathogens in patients with chronic periodontitis: correlation with clinical indices

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Kinane DF, Mooney J, MacFarlane TW, McDonald M. Local and systemic antibody response to putative periodontopathogens in patients with chronic periodontitis: correlation with clinical indices.
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Specific immunoglobulin G (IgG), IgA and IgM antibody titres to *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* were measured by enzyme-linked immunosorbent assay in serum and gingival crevicular fluid at 5 sites in each of 20 chronic periodontitis patients. Specific serum antibody titres correlated with mean gingival crevicular fluid titres. The 3 immunoglobulin subclass responses (IgA, IgG and IgM) to *P. gingivalis* correlated. A comparison of sites with probing depth <4 mm and ≥ 4 mm showed that the latter group had significantly lower gingival crevicular fluid IgG titres to *P. gingivalis*. Sites with a gingival index of 3 had significantly lower gingival crevicular fluid IgG titres to this organism than those with a gingival index of less than 3. These findings support the concept that the humoral immune response is protective, as chronic periodontitis patients with greater pocket depths and more gingival inflammation had paradoxically lower antibody titres to suspected periodontopathogens.

Key words: antibody; *Porphyromonas gingivalis; Actinobacillus actinomycetemcomitans;* ELISA; gingival crevicular fluid; chronic periodontitis

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Porphyromonas gingivalis and *Actinobacillus actinomycetemcomitans* are considered important periodontopathogens in different types of periodontal disease (21, 22). Studies of the systemic humoral immune response to these organisms have related specific serum antibody titres to disease progression. For example, Gunsolley et al. (12) found that the level of serum antibody to *P. gingivalis* and *A. actinomycetemcomitans* in periodontitis subjects was inversely related to disease level. Ebersole et al. (7, 8), however, reported a positive correlation between serum immunoglobulin G (IgG) to *A. actinomycetemcomitans* and the severity of the disease. Also when patients with similar *P. gingivalis* antibody levels were compared, greater disease severity was evident in rapidly progressive periodontitis patients than in adult periodontitis and localized juvenile periodontitis patients (7).

The relationship between local antibody levels and local disease status has not been investigated to the same extent. Baranowska et al. (1) found no significant difference in the level of specific IgG to *P. gingivalis* in gingival crevicular fluid (GCF) between healthy and diseased sites within the same individual. Tew et al. (24) found no obvious differences in the clinical parameters of probing depth and attachment level between sites with elevated antibody to *P. gingivalis* and/or *A. actinomycetemcomitans* and those with normal or low levels. They concluded that elevated antibody in GCF may relate to changes in disease activity that are not detectable by conventional clinical assessment techniques. Suzuki et al. (23) demonstrated that local production of IgG to *P. gingivalis* was markedly increased in adult periodontitis as compared with rapidly progressive periodontitis, suggesting that disease progression is influenced by local antibody production. Challacombe et al. (2) showed that levels of IgG antibody to *P. gingivalis* were lower in crevicular fluid washings of patients with a high periodontal disease index than in those with low scores (not statistically significant). In addition, the opsonic activity against *P. gingivalis* was significantly depressed in high periodontal disease index patients as compared with low-scoring patients.

Microbial challenge generally induces antibody production and high titres indi-

cate current or previous infection. However, in periodontal disease the correlation between high antibody titres and disease status is equivocal. The aims of this study were to measure the local (GCF) and systemic (serum) levels of IgG, IgA and IgM antibody to type strains of *P. gingivalis* and *A. actinomycetemcomitans* within a group of 20 periodontitis patients and to correlate these with pocket depth and gingival index.

Material and methods

Patients

The subjects were 20 patients with moderate periodontitis who were recall maintenance therapy and had previously undergone active periodontal treatment. All patients were over 18 years of age with no history of systemic disease or history of antibiotic therapy within the previous 3 months. Five non-adjacent periodontal sites, previously selected by radiograph as being the most severely affected (greatest loss of periodontal bone), were sampled. These sites varied in disease status as defined by probing depth (PD) (18) and modified gingival index (GI) (18). Gingival crevicular fluid was collected on Whatman grade 4 filter paper strips (11) over a period of 30 s. Strips contaminated by blood were discarded. Fluid volume was quantified by a Periotron 6000 (Harco Electronics, Winnipeg, Manitoba, Canada). The strips were placed in vials containing 1 ml of phosphate-buffered saline (PBS) with 0.1% bovine serum albumin and 0.05% Tween 20 and antibody was eluted for 1 h with mixing at 25°C. The eluates were aliquotted and stored at −20°C until used. Serum was also collected from each patient and stored at −20°C.

Clinical sampling technique

Sites for analysis were chosen on the basis of radiographic evidence and the order of sampling and clinical assessment was as follows. The modified GI (non-invasive) was recorded followed by careful supragingival debridement. GCF was sampled next, and finally pocket depth was recorded.

Enzyme-linked immunosorbent assay (ELISA)

Specific antibody titres were measured by ELISA based on the method of Ebersole et al. (4, 5), using formalinized whole cells at an absorbance (OD 600) that had previously been determined as optimum to coat microtitre plates. The bacterial strains used were *P. gingivalis* NCTC 11834, *A. actinomycetemcomitans* ATCC 29523 and *Haemophilus aphrophilus* NCTC 5886. *P. gingivalis* was grown under anaerobic conditions (85% N_2, 10% H_2, 5% CO_2) and *A. actinomycetemcomitans* in CO_2 at 37°C on Columbia blood agar. *P. gingivalis* was harvested after 5 days and *A. actinomycetemcomitans* after 24 h into PBS (pH 7.4), with 1 mM disodium EDTA, washed by centrifugation, and fixed for 1 h in 10% formal saline. The cells were then washed twice in PBS and once in 0.1 M sodium carbonate-bicarbonate buffer containing 0.02% NaN_3 at pH 9.6 (coating buffer). Fixed cells were stored in coating buffer at 4°C until use.

Immulon 1 plates (Dynatech Industries, McLean, VA) were used because of their low protein-binding characteristics. After coating, the plates were treated with PBS containing 0.1% bovine serum albumin, 0.05% Tween 20 and 5% skimmed milk to remove background binding. Serum or GCF diluted in this buffer to a concentration within the range of the calibration graph was then added for 2 h at 37°C, and the plates were subsequently incubated with biotin-anti-human IgG (150 ng/ml), IgA (220 ng/ml) or IgM (170 ng/ml) (Sigma Chemical Co., Detroit, MI) and thereafter with 1 μg/ml extravidin-peroxidase (Sigma Chemical Co.).

The reaction was visualized using o-phenylenediamine substrate and stopped with 1 M H_2SO_4. The optical densities were read at 490 nm. The samples were assayed in triplicate, correction was made for non-specific binding and the results were read from a reference line derived from serial dilutions of a reference positive control serum. The results were expressed as ELISA units (10).

Adsorption studies

Sera were prediluted 1/200 in PBS and were added to an equal volume of bacterial suspension at an OD_{600} related to the optimum coating concentration for that organism (for *A. actinomycetemcomitans* the OD_{600} was 0.5 and for *H. aphrophilus* the OD_{600} was 0.8). These were mixed at 37°C for 6 h and then incubated at 4°C overnight. The sera were centrifuged and the supernatant collected for subsequent analysis.

Statistical analysis

The correlations between serum and GCF were assessed by Spearman rank-order correlation analysis. The comparisons between groups of sites were performed using Mann-Whitney tests for nonparametric data. Antibody titres were generally expressed as ELISA units per 30-s sample to eliminate inaccuracies caused by calculations involving very low or high volumes of GCF beyond the limits that can be accurately recorded by the Periotron (17). In some cases, where direct comparisons between serum and GCF were made, antibody titres were expressed as ELISA units/ml.

Results

The Mann-Whitney tests relating the sites grouped on PD and GI with local IgG antibody levels to *P. gingivalis* and *A. actinomycetemcomitans* are presented in Table 1. *P. gingivalis* IgG levels were found to be lower in the sites with PD greater than or equal to 4 mm as compared with PD < 4 groups ($P < 0.01$) and GI ≥ 3 as compared with GI < 3 groups ($P < 0.001$). Further tests relating the sites grouped on PD and GI with IgG antibody to *A. actinomycetemcomitans* (Table 1) or IgA antibodies to *P. gingivalis* gave no statistically significant differences for these comparisons.

Table 1. Comparisons between different degrees of PD and GI and IgG antibodies in GCF to *P. gingivalis* and *A. actinomycetemcomitans* at these sites (Mann-Whitney test)

	P. gingivalis IgG (EU/30 s)				*A. actinomycetemcomitans* IgG (EU/30 s)			
	n	mean	(SD)		*n*	mean	(SD)	
PD < 4	45	891	(2304)	$P < 0.01$	17	25	(46)	NS
PD ≥ 4	49	518	(1775)		32	21	(46)	
GI < 3	61	1602	(2465)	$P < 0.001$	36	21	(43)	NS
GI ≥ 3	28	24	(67)		8	41	(64)	

NS = not significant. Comparisons for all sites were not performed due to the limited volume of GCF available in site samples. IgA response to *P. gingivalis* was also tested but gave no significant differences between clinical index groupings. EU = ELISA units.

Table 2. Correlations between serum and mean GCF antibodies

			Mean (EU/ml)			
	n	*r*	Serum	GCF	R^2	*P*
P. gingivalis IgG	16	0.82	2987	512	66.7%	<0.001
P. gingivalis IgA	13	0.22	265	157	6.8%	NS
A. actinomycetemcomitans IgG	10	0.98	2738	121	96.3%	<0.001

NS = not significant. Correlations of *P. gingivalis* IgM and *A. actinomycetemcomitans* IgM and IgA were not performed due to the limited volume of gingival crevicular fluid available in site samples. EU = ELISA units.

Table 2 shows the correlations between serum and mean GCF levels for IgG and IgA against *P. gingivalis* and IgG directed against *A. actinomycetemcomitans*. Both *P. gingivalis* and *A. actinomycetemcomitans* IgG serum and GCF levels correlated well (R^2 = 66.7% and 96.3% respectively; $P < 0.001$). Reanalysis of the data categorized on clinical indices revealed slightly higher R^2 values for the sites with more disease (i.e. GI = 3 and PD ≥ 4 mm) but did not show a significant trend. Due to the restrictions imposed by the limited sample volume (mean = 0.31 μl) obtained using the standardized 30-s GCF sampling technique, insufficient sample was available to compare via ELISA, specific GCF IgM with IgA and IgG. Previous work within our laboratory has indicated that levels of total and specific IgM in GCF were much lower than both IgA and IgG.

The correlations between serum IgG, IgA and IgM levels to *P. gingivalis* were examined. The 3 serum immunoglobulin class responses to *P. gingivalis* correlated well. Similar correlations for *A. actinomycetemcomitans* and *H. aphrophilus* did not occur within the main antibody classes, although correlations between *H. aphrophilus* and *A. actinomyctemcomitans* IgG and IgM were highly significant ($P < 0.001$), which indicates that these species may share cross-reacting antigens. Adsorption studies were carried out in which the 20 patients' sera were adsorbed with either *A. actinomycetemcomitans* or *H. aphrophilus*. The adsorbed sera were then assessed for specific antibody to *A. actinomycetemcomitans*. *A. actinomycetemcomitans* adsorption resulted in a mean reduction in anti-*A. actinomycetemcomitans* IgG of 68% (SD = 21%), and a similar reduction of 63% (SD = 22%) was achieved following adsorption with *H. aphrophilus*. These results give additional evidence for cross-reactivity between antibodies to these 2 bacterial species in chronic periodontitis patients.

Discussion

Specific IgG levels against *P. gingivalis* were found to be significantly lower in more severe chronic periodontitis sites as defined by PD and GI. However, no significant differences in IgA levels were found between chronic periodontitis sites of varying disease levels (Table 1). Baranowska et al. (1) found no difference between levels of specific IgG to *P. gingivalis* in healthy and diseased sites within the same individual. However, fixed volume samples of 0.5 μl were taken. This means that strips from sites that did not yield 0.5 μl were discarded and that samples were taken over variable time periods, resulting in variable rates of serum contamination (3). Since healthy sites tend to yield lower volumes than diseased sites, this method treats healthy and diseased sites in different ways. Therefore, these results are not strictly comparable with the present study. An earlier study by Schenck (20) showed an inverse relationship between the number of deep pockets (>4 mm) and serum antibody level to *P. gingivalis* lipopolysaccharide in chronic periodontitis patients. Mouton et al. (19) demonstrated a dichotomy in serological responses to *P. gingivalis* among chronic periodontitis patients, with one subgroup exhibiting high serum antibody levels and another having levels similar to those of healthy individuals. The patients with high serum antibody levels appeared to respond better to therapy, as evidenced by reduction in the number of deep pockets within one month post-treatment. Their findings are consistent with the data reported here than lower *P. gingivalis* IgG titres are found in more severely affected sites.

A fairly strong correlation between IgG, but not IgA, to *P. gingivalis* in serum and GCF was found in this study (Table 2), and similar results have been reported previously (1). However, other studies have reported lower mean GCF than serum IgG levels to *P. gingivalis* (5) as well as lower total IgG in GCF than in serum (13). In addition, Lamster et al. (16) reported a significant correlation between total IgG in GCF and specific serum antibody to *Bacteroides intermedius* but not *P. gingivalis*. They conclude that the development of a serum immunoglobulin response to suspected periodontopathogens is consistent with a protective host response. A corollary of this view is that a local deficiency of IgG to *P. gingivalis* may lead to local disease progression. Alternatively, Kilian (15) has demonstrated that *P. gingivalis* can degrade human IgG and IgA, suggesting that low GCF levels of IgG may be caused by degradation by *P. gingivalis*, or that locally available antibodies are adsorbed by the greater mass of subgingival plaque present.

Levels of serum and GCF IgG against *A. actinomycetemcomitans* were found to be highly correlated in this study. In contrast to the results presented on *P. gingivalis* IgG, there were no differences in GCF IgG levels to *A. actinomycetemcomitans* in chronic periodontitis sites of varying disease levels (Table 1). Gunsolley et al. (12) found that serum antibody levels to *A. actinomycetemcomitans* and *P. gingivalis* are inversely related to the degree of periodontal destruction in young adults with juvenile periodontitis or generalized severe periodontitis and concluded that this is consistent with a hypothesis that failure to mount a substantial antibody response to these organisms leads to more widespread periodontal destruction. The present study of adult periodontitis patients would support this view at both local and systemic levels, but only for *P. gingivalis*.

Genco et al. (9) showed that adsorption of serum from localized juvenile periodontitis patients with *A. actinomycetemcomitans* and *H. aphrophilus* resulted in the elimination of most of the reactivity to the homologous strain but not to the heterologous strain. In the present study, most of the reactivity to *A. actinomycetemcomitans* could be removed by adsorption with both organisms, suggesting that there is cross-reaction between antibodies to these 2 species. Similarly, strong correlations were found between serum IgG and IgM levels to these 2 organisms. The indications are that antibodies detected to *A. actinomycetemcomitans* in adult periodontitis patients could be confounded with cross-reacting antibodies directed against *H. aphrophilus*, which is, with

other haemophili, commonly isolated from dental plaque (14).

Ebersole et al. (6) demonstrated in patients with various types of periodontal disease that the same organism to which the individual exhibited elevated serum antibody was detected in 55% of disease-active sites but only 18% of disease-inactive sites. However, in a study of patients with early-onset periodontitis, Williams et al. (25) found that many patients had serum antibodies to organisms not found in the pocket flora and that some organisms that made up a large proportion of the pocket flora did not seem to provoke a corresponding antibody response. These authors suggested that sequential infection occurs in these forms of periodontitis, leading to induction of protective immunity against reinfection by the same organism.

In conclusion, there appears to be general agreement that antibody levels to *P. gingivalis* are elevated in periodontitis patients as compared with healthy individuals, but there are also indications, supported by the present study, that lower antibody levels may be present in the more affected sites within the periodontitis group. In addition, this study failed to demonstrate any significant differences between GCF IgG levels against *A. actinomycetemcomitans* between sites with different PD and GI. Care must be taken, however, in interpreting these results, as although these patients exhibited sites with pocketing and gingivitis (Table 1), they were maintenance regimen patients who have received extensive periodontal treatment. Thus, although they may not be strictly comparable with non-treated adult periodontitis patients, this study addresses the cross-sectional comparison of healthy and diseased sites and the correlation between local and systemic antibody titres. Further work is required to elucidate the inverse relationship between the clinical indices and GCF and serum IgG antibody to *P. gingivalis* found in the periodontitis patients of the present study.

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Relative avidity of serum antibodies to putative periodontopathogens in periodontal disease

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ELISA was used to determine both the avidity and titre of IgG, IgA and IgM antibodies to the gram-negative anaerobe, *Porphyromonas gingivalis*, in twenty periodontitis patients enrolled in a longitudinal study of attachment loss and eleven non-periodontitis affected subjects. The avidity and titre of IgG antibodies to *Actinobacillus Actinomycetemcomitans* were also examined. A cross-sectional analysis of the longitudinal patients at baseline and non-periodontally affected controls confirmed earlier findings that IgG and IgA antibody titres to *P. gingivalis* were higher in periodontitis patients than in individuals who were not periodontally affected. In this cross-sectional analysis, IgG antibody avidities to *P. gingivalis* were not found to be significantly higher in periodontitis than in control subjects ($p=0.065$). However, indications of the potential prognostic value of antibody avidity was demonstrated by the higher IgM avidities to *P. gingivalis* in patients who did not experience attachment loss during the three-month monitoring period than in those who did ($p=0.0005$).

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Key words: relative avidity; *P. gingivalis*; *A. actinomycetemcomitans*; periodontitis; serum antibodies

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Previous studies have reported antibodies directed against putative periodontopathogens, e.g. *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans*, in the sera of patients with periodontal disease and also in healthy subjects (1, 2). Controversy surrounds the exact relationship between antibody titres to these organisms and disease progression. For example, Gunsolley *et al.* (3) found that the level of serum antibody to these two organisms in periodontitis subjects was inversely related to disease level. Ebersole *et al.* (4, 5), in contrast, reported a positive correlation between serum IgG to *A. actinomycetemcomitans* and the severity of the disease.

These studies have tended to examine titres of antibodies and there has been little work on antibody avidity in relation to periodontal disease. Avidity is a measure of the net binding strength between antibodies and antigens. Bacteria normally have numerous antigens on their surface and each of these may have several epitopes capable of provoking an antibody response. The antibodies produced in response to each epitope will be of differing affinities. The innate intrinsic strength of the interaction between a single epitope on the antigen and an antigen-binding site on the antibody is defined as the affinity of that interaction. The avidity of serum antibodies to microorganisms involves many antigenic and epitopic interactions. Therefore, avidity in this context is an overall measure of the mean affinity or overall stability of potentially extensive interactions.

Lopatin *et al.* (6) showed that the avidity of IgG-class antibodies to *P. gingivalis* was significantly increased in periodontitis patients compared with healthy controls. No such elevation was detected in IgM-class antibodies. The authors suggested that since human antibodies to *P. gingivalis* appeared to be of generally low avidity, compared with those in rabbits immunized with this organism, the pres-

Abbreviations: Ig – Immunoglobulin; ELISA – Enzyme linked immunosorbent assay; EDTA – ethylene diamine tetracetic acid; PBS – phosphate buffered saline; BSA – bovine serum albumin; ssdna – single strand DNA.

ence of low avidity antibodies may contribute to the pathology associated with periodontal disease.

Chen *et al.* (7) demonstrated that IgG avidities to *P. gingivalis* were lower in rapidly progressive periodontitis (RPP) patients than in control subjects. However, after treatment the avidities increased significantly to levels higher than in controls. They concluded that many RPP patients do not produce protective levels of biologically functional antibody as a result of natural infection, but treatment may induce the production of such antibodies.

This study examined the relationship between antibody avidity and periodontal status. Titres and avidity of three immunoglobulin classes (IgG, IgM and IgA) against *P. gingivalis* and of IgG to *A. actinomycetemcomitans* were assayed in a cross-sectional comparison of adult periodontitis patients on maintenance and healthy controls. The association of avidity with probing attachment loss in the course of a three month duration longitudinal study and the relationship between avidities and titres of antibodies to *P. gingivalis* and *A. actinomycetemcomitans* was also examined.

Material and methods

Bacteria

P. gingivalis NCTC 11834 was grown under anaerobic conditions (85% N_2, 10% H_2, 5% CO_2) and *A. actinomycetemcomitans* in CO_2 at 37°C on Columbia blood agar. *P. gingivalis* was harvested after 5 days and *A. actinomycetemcomitans* after 24 h into phosphate-buffered saline, 1 mM Na_2 EDTA, pH 7.4 (PBSE), washed by centrifugation, and fixed for 1 h in 10% formal saline. The cells were than washed twice in PBSE and once in 0.1 M Na carbonate-bicarbonate buffer containing 0.02% NaN_3 at pH 9.6 (coating buffer). Fixed cells were stored in coating buffer at 4°C until use.

Subjects

Serum was collected from a total of 31 subjects. These included 20 adult periodontitis patients (age range: 28–68 years; 11 men, 9 women) and 11 periodontally healthy individuals (age range: 22–35 years; 6 men, 5 women).

The 20 periodontitis patients of the present study participated in a 3-month duration longitudinal study of periodontal disease activity which actually followed 38 patients in total who had been diagnosed in the past as suffering from advanced periodontal disease (at least one site demonstrating probing depth >6 mm in at least four sextants). These patients had received a full course of periodontal treatment in the Glasgow Dental Hospital, were on maintenance for at least 1 year and demonstrated pocketing of greater or equal to 4 mm in at least four non-adjacent sites, as assessed during conventional periodontal charting using the PC12 periodontal probe with a tip diameter of 0.4 mm (Prisme, U.K.). In addition, these patients had not received any antibiotics for 3 months prior to the initiation of this study. All non-adjacent sites, demonstrating probing depth above or equal to 4 mm, were monitored in these 38 patients over a 3-month period. A minimum of 6 and a maximum of 15 non-adjacent sites were thus followed per subject, and a full clinical data set including duplicate attachment level measurements and gingival crevicular fluid samples was taken. In addition, serum samples were taken at the baseline and three month appointments. Attachment level change was determined using a custom-made soft acrylic stent, the Florida probe stent handpiece (Florida Probe Corporation, Florida, USA) (8), and the tolerance method (9). The full clinical data set of the 38 patients and the analysis of gingival crevicular fluid will be presented elsewhere.

Fourteen of these 38 periodontitis patients, who constituted the attachment loss group (AL) of subjects in the present study, demonstrated significant attachment loss in at least one site during the 3-month observation period.

A subgroup of the remaining 24 periodontitis patients, who did not demonstrate significant attachment loss at the studied sites, was made by retrospective examination of previous periodontal pocket chartings. This subgroup of 6 'stable' subjects was selected to form the non-attachment loss group (NAL) in the present investigation. These 6 patients did not demonstrate attachment loss greater or equal to 2 mm at any site, as assessed by manual periodontal probing using the PC12 periodontal probe (Prisma, U.K.) over the whole period of their periodontal maintenance (a minimum of 1 year) during the normal recall system of the Periodontal Department of the Glasgow Dental Hospital. In contrast, the remaining 18 subjects had demonstrated progressive attachment loss of greater than 2 mm according to conventional periodontal pocket chartings in at least one site within the last year of periodontal maintenance. These 18 patients were therefore not considered 'stable' and were excluded from the NAL group in the present immunological study on relative antibody avidity.

The periodontally healthy individuals did not demonstrate significant gingival inflammation or attachment loss at any site. In addition, pocketing was below 3 mm at all sites. A serum sample was obtained from each healthy control subject at one time-point in a cross-sectional manner. When cross-sectional comparisons involving the 20 perio-

dontitis subjects were made, data from baseline sera (0 months) were used.

ELISA

Specific antibody titres were measured by ELISA based on the method of Ebersole *et al.* (10), using formalinized whole cells at an absorbance which had previously been determined as optimum to coat microtitre plates. Immulon 1 plates (Dynatech) were employed because of their low protein-binding characteristics. After coating, the plates were treated with PBS containing 0.1% bovine serum albumin (BSA), 0.05% Tween 20 and 5% skimmed milk to reduce non-specific antibody binding to the plates. Serum serially diluted in this buffer was then added and the plates were subsequently incubated with biotin-conjugated anti-human IgG, IgA or IgM (Sigma) and thereafter with ExtrAvidin-perioxidase (Sigma). Reaction was visualized using o-phenylenediamine dihydrochloride substrate and stopped with 1 M H_2SO_4. Samples were assayed in duplicate and results were calculated using a regression line and derived equation from serial dilutions of a reference serum. Results were expressed as ELISA units (EU) (11).

Dissociation assay

The dissociation assay to determine antibody avidity was performed as follows: after incubation with serum as described above, the wells were treated with increasing concentrations of ammonium thiocyanate (0.2–8.0 M). The concentration of thiocyanate required to dissociate 50% of bound antibody was determined by linear regression analysis. This was termed the ID_{50} and provides a measure of relative avidity as previously reported (12, 13).

Statistical analysis

Two-sample t-tests were used to determine differences in avidity between different clinical groups. Mann-Whitney tests were used in group comparisons of the non-normally distributed antibody titres. Student's paired t-tests were used to assess whether differences were significant between baseline and recall appointments and to compare relative avidities of antibodies to *P. gingivalis* and *A. actinomycetemcomitans* in various sera.

Results

The periodontally healthy (n = 11) and maintenance periodontitis groups (n = 20) were compared in terms of titre and avidity of IgG, IgM and IgA class antibodies to *P. gingivalis*. The periodontitis group was also sub-divided into a sub-group of 14 patients who experienced attachment loss in at least one site during the 3-month monitoring period (AL) and another sub-group of 6 patients who exhibited no attachment loss (NAL). Table 1 shows the median titres and mean avidities of the three classes of antibodies in controls and perio-

Table 1. Titres and avidities of antibodies to *P. gingivalis* in control subjects and total periodontitis patients and periodontitis patients grouped according to attachment loss and no attachment loss

	Controls (n = 11)		Periodontitis patients					
			Total (n = 20)		Attachment loss (n = 14)		No attachment loss (n = 6)	
	Median titre (EU)	Mean avidity (M)	Median titre (EU)	Mean avidity (M)	Median titre (EU)	Mean avidity (M)	Median titre (EU)	Mean avidity (M)
IgG	320 (107–884)[1]	1.21 (0.85–1.70)	2390 (1017–5595)[1]	1.74 (1.12–2.14)	2390 (841–4806)	1.66 (1.21–2.04)	2149 (1017–7835)	1.93 (0.95–3.34)
IgM	537 (188–2170)	0.81 (0.69–0.94)	206 (105–603)	0.78 (0.64–0.93)	280 (105–761)	0.70[3] (0.60–0.84)	153 (72–722)	0.97[3] (0.86–1.07)
IgA	28 (19–60)[2]	1.35 (0.60–1.74)	586 (151–1478)[2]	1.66 (1.34–1.99)	631 (44–2261)	1.66 (1.16–2.01)	268 (187–14154)	1.66 (1.37–2.04)

[1,2,3] Significant differences between compared groups are shown by paired symbols ($p < 0.01$). Interquartile ranges (Q_1–Q_3) given in parentheses.

Table 2. Titres and avidities of antibodies to *P. gingivalis* for all periodontitis subjects (n = 20) at baseline and after three months

	Median titre (EU)				Mean avidity (M)			
	Baseline	3 months	Mean difference	Interquartile range	Baseline	3 months	Mean difference	Interquartile range
IgG	2390	1824	−566	−603/+1400	1.74	2.02	+0.28	−0.16/+0.73
IgM	206	150	−56	−115/+328	0.78	0.70	−0.08	−0.29/+0.11
IgA	586	410	−176	−194/+441	1.66	1.49	−0.17	−0.51/+0.32

No significant differences were detected between baseline and 3 months for all comparisons: Student's paired t-test.

dontitis patients at baseline. Comparisons between patients and controls for titre and avidity to *P. gingivalis* in all three immunoglobulin classes were made and significant differences ($p < 0.01$) were only noted between IgG median titres and IgA median titres (Mann-Whitney or two-sample t-test). The comparison between periodontitis patients at baseline grouped in terms of attachment loss and lack of attachment loss after three months is also shown in this table. A significant difference was noted between the mean avidity of IgM antibodies between subjects with attachment loss and those without attachment loss ($p < 0.001$).

Table 2 shows the changes in antibody titre and avidity to *P. gingivalis* between baseline and three months for all periodontitis subjects but indicates no significant differences. Interestingly, sub-grouping the periodontitis patients into AL and NAL gave divergent results for IgG avidity to *P. gingivalis*, in that over the 3-month period, the AL group had avidities which tended to increase whereas the NAL avidities tended to decrease (Table 3). Similarly, although also not statistically significant, IgM and IgA avidities tended to decrease more in NAL than in AL patients.

Healthy control and periodontitis groups at baseline were compared for differences in IgG titre and avidity to the two organisms (*P. gingivalis* and *A. actinomycetemcomitans*) and the results are shown in Table 4. These show that avidities to *P. gingivalis* were significantly higher than to *A. actinomycetemcomitans* in both groups but only in the periodontitis group was this difference significant.

Table 5 presents the correlations between titre and avidity. These correlations (titre to avidity) are shown for IgG, IgM and IgA specific for *P. gingivalis* and IgG specific for *A. actinomycetemcomitans*. The subject groups for which these are shown are: all subjects (n = 31); control subjects (n = 11); all periodontitis subjects (n = 20); attachment loss periodontitis subjects (AL) (n = 14); and non-attachment loss periodontitis subjects (NAL) (n = 6). Baseline data were used for the periodontitis patients. The results of the correlation analyses are shown as R^2 (%) and p-value. The data presented in Table 5 show that there was a significant correlation between titre and avidity of IgG against *P. gingivalis* for NAL patients and between titre and avidity of IgM against *P. gingivalis* for AL patients.

Table 3. Longitudinal changes in avidities of IgG antibodies to *P. gingivalis* for AL and NAL periodontitis subjects

		Mean change in avidity (Baseline-3 months)	p-value (Student's paired t-test)
IgG	AL (n = 14)	+0.46 (−0.13/+1.12)	0.14
	NAL (n = 6)	−0.10 (−0.47/+0.42)	0.74
IgM	AL (n = 14)	−0.01 (−0.14/+0.11)	0.80
	NAL (n = 6)	−0.29 (−0.60/−0.09)	0.22
IgA	AL (n = 14)	−0.07 (−0.54/+0.35)	0.71
	NAL (n = 6)	−0.15 (−0.74/−0.61)	0.74

Interquartile ranges (Q_1–Q_3) given in parentheses.
AL: Attachment loss periodontitis group.
NAL: Non-attachment loss periodontitis group.

Discussion

The relationship between antibody titres to various suspected periodontopathogens and periodontal status has been extensively investigated although many of the findings have been contradictory and this remains a highly controversial field. Ebersole & Holt (5) have dealt with the application of antibody titres to diagnostics in great detail. However, Wilton *et al.* (2), in their review, stated that the data currently available does not allow these responses to be diagnostic. Generally, reports in this field are agreed that an elevation in the humoral immune response to certain plaque-associated microorganisms is detected in the presence of and/or increasing extent of periodontitis (14, 15). However, a few studies have suggested the opposite tendency (16, 17).

Antibody avidity, that is the measure of the net binding strength of antibody to antigen, has been extensively studied in a number of fields, both in relation to antibody titre and in isolation, in terms of disease susceptibility and progression. For example, Morikawa *et al.* (18) found that the titre of IgA to soybean antigen in the breast milk of Indian mothers was significantly higher than in Japanese mothers, although the avidity was significantly lower. Udhayakumar *et al.* (19) have shown that monoclonal antibodies of higher avidity have much greater effectiveness in activating B-cells and presumably enhancing an immune response. Interestingly, Charoenvit *et al.* (20) found that a monoclonal antibody to *Plasmodium yoelii*, a malarial parasite, has lower avidity for the antigen than vaccine-induced polyclonal antibodies in mice. Doi *et al.* (21) suggested that low avidity may be a pathogenic characteristic of IgG circulating immune complexes in membraneous nephropathy. Significantly, Joynson *et al.* (22) found that toxoplasmosis patients with acute infection had low avidity IgG whereas those with chronic infection had high avidity. This may have application in investigations of the phasic destructive episodes of

Table 4. Titres and avidities of IgG antibodies to *P. gingivalis* and *A. actinomycetemcomitans* (A.a.) for control and periodontitis subjects at baseline

	Control (n = 11)		Periodontitis (n = 20)	
	P. gingivalis	*Aa*	*P. gingivalis*	*Aa*
IgG Median titre	320 (107–884)	173 (141–214)	2390 (1017–5595)	1189 (109–12239)
IgG Mean avidity	1.21 (0.85–1.70)	0.93 (0.73–1.03)	1.74* (1.12–2.14)	0.83* (0.50–0.94)

* Significant difference: $p < 0.01$ for comparison (Student's paired t-test).
Interquartile ranges (Q_1–Q_3) given in parentheses.

Table 5. Correlations between titres and avidities. R^2 (%) and associated p-values are given for all immunoglobulin classes for *P. gingivalis* and IgG antibodies for *A. actinomycetemcomitans* (*A.a.*) in each subject group

		P. gingivalis			*A.a.*
		IgG	IgM	IgA	IgG
All subjects	n = 31	42.0%, <0.001*	10.2%, 0.079	7.5%, 0.257	24.3%, 0.009*
Control subjects	n = 11	24.3%, 0.123	16.5%, 0.215	0.1%, 0.939	−1.2%, 0.762
All periodontitis subjects	n = 20	39.2%, 0.003*	12.2%, 0.131	1.4%, 0.689	32.2%, 0.018*
AL	n = 14	13.7%, 0.193	54.9%, 0.002*	14.1%, 0.284	90.1%, <0.001*
NAL	n = 6	69.3%, 0.04*	12.6%, 0.491	18.2%, 0.474	0.3%, 0.932

Results given as R^2 (%), p-value. * denotes significant correlations (significance level of $p < 0.05$).
AL = Attachment loss periodontitis group.
NAL = Non-attachment loss periodontitis group.

chronic periodontitis, with the possibility that acute exacerbations and quiescent periods could be differentiated. The secondary immune response classically generates antibodies of higher avidity. Francus *et al.* (23) have demonstrated that a possible mechanism may be carrier primed T-cells which selectively activate virgin B-cells, which are then committed to the production of high avidity antibodies. A study with particular relevance to the present report is that of Panoskaltsis and Sinclair (24) who showed that autoimmune mice have lower avidity anti-ssDNA (single-strand DNA) IgM antibodies than non-autoimmune mice.

There have, however, been very few reports dealing with antibody avidity in relation to periodontal disease. Ebersole *et al.* (25) studied the increase in avidity in the non-human primate, *Macaca fascicularis*, following immunization with tetanus toxoid, which they used as a prototype bacterial exotoxin. They found that IgG avidity increased from 0.9 M to 1.72 M following primary immunization and 2.56 M after secondary immunization. Lopatin *et al.* (6) demonstrated that avidity of antibody rose to a similarly high level in rabbits post-immunization with *P. gingivalis*, but that human antibodies to this organism appear to be of generally low avidity. This latter finding was confirmed by the present study. The present report also confirms the finding of Lopatin *et al.*, and others (26, 27, 28), that IgG serum antibody titres to *P. gingivalis* are significantly higher in periodontitis patients on maintenance than in controls ($p < 0.01$) (Table 1). Additionally we show that this is also the case for IgA antibodies, but not IgM. However, we were not able to demonstrate a statistically significant difference in antibody avidity for any immunoglobulin class in the above subject groups. The observation that IgM avidity to *P. gingivalis* is higher in NAL than in AL patients is difficult to explain, although perhaps the metabolic block referred to by Panoskaltsis and Sinclair (24) preventing IgM-IgG switching and leading to increased IgM avidity is operating here. Nevertheless, the finding may have prognostic significance if confirmed by further studies.

Chen *et al.* (7) concluded that after periodontal treatment of rapidly progressive periodontitis patients, their IgG antibody avidity increased. The data from the present study presented in Table 5 may reflect the treatment effect reported by these authors. In this case the treatment effect would generate a strong and statistically significant positive correlation between titres and avidities of antibodies to *P. gingivalis* in NAL patients as seen in

Table 5. This would result from increased avidities and decreased titres stimulated by treatment, or in other words, a shift to more biologically effective antibodies. This patient sub-group may in fact correspond to the "seropositive" subgroup of Chen *et al.*'s study. However, the present study dealt with adult periodontitis patients in maintenance phase and Chen *et al.* examined rapidly progressive periodontitis patients prior to, and after initial treatment, therefore the two studies are not strictly comparable.

Another recent study of titre and avidity of IgG antibodies to *P. gingivalis* in rapidly progressive periodontitis (RPP) by Whitney *et al.* (29) also shows lower avidities in RPP patients than in controls. This contrasts with our study of adult periodontitis patients which demonstrated a trend towards higher IgG avidities in patients than in healthy controls ($p=0.065$). Moreover, if seropositive healthy control subjects are excluded using the criterion of Chen *et al.* (7), i.e. titre $>2\times$ median control titre, then this yields a significant difference between seronegative control subjects and adult periodontitis patients ($p=0.02$). These differences between the periodontitis patient groups may reflect a differing immunological susceptibility.

The longitudinal aspect of the study failed to show any significant changes in titre or avidity of antibodies to *P. gingivalis* during the 3-month monitoring period (Table 3). Although not statistically significant, the increase in avidity of 0.46 M in AL patients compared with a decrease of 0.10 M for NAL patients ($p=0.14$) may suggest long-term changes in antibody avidity which should be examined further by longitudinal studies.

The comparison of the responses to *P. gingivalis* and *A. actinomycetemcomitans* is interesting in that the avidity of IgG antibodies to the latter was found to be significantly lower than to the former (Table 4). However, only the periodontitis group were seen to have a significant divergence in response (i.e. equivalent to 6–7 times the binding strength) (Table 4). The relevance of this is unclear but there is a suggestion that not only do IgG avidities to *P. gingivalis* tend to be higher in periodontitis, but IgG avidities to *A. actinomycetemcomitans* tend to be lower than normal as shown in Table 4. This may indicate the differing relevance of these two organisms in adult periodontitis.

A very recent study by Sjostrom *et al.* (30) showed that IgG antibodies in low-titre sera from control subjects were significantly more effective in opsonizing *A. actinomycetemcomitans* than IgG antibodies in low-titre sera from RPP patients. The finding of the present study that avidities of IgG antibodies to *A. actinomycetemcomitans* tend to be lower in adult periodontitis patients than in healthy control subjects, although this failed to reach statistical significance, may indicate a failure of biological function of antibody directed against *A. actinomycetemcomitans* in these patients also.

Unlike Lopatin *et al.* (6) we have found a significant correlation between titre and avidity of the IgG response to both organisms studied. In addition, our data show that there are differences between patients groups in relation to these correlations. The data presented in Table 5 suggest that switching to higher avidity IgG antibodies may be occurring in those patients who tend not to experience further attachment loss, but not in those who do. This indicates the potential prognostic value of antibody avidity in determining patients susceptibility to further attachment loss.

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Immunocytochemical characterization of cellular infiltrate, related endothelial changes and determination of GCF acute-phase proteins during human experimental gingivitis

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Introduction

During the initiation of inflammation, vascular endothelium is activated by a variety of molecules which increase the permeability of vessels and regulate the passage of leukocytes from the bloodstream into the inflamed tissues. Of the molecules capable of acting on the endothelial cells, interleukin-1 (IL-1), an inflammatory cytokine, has been detected in inflamed gingiva (1). IL-1 upregulates expression of endothelial leucocyte adhesion molecules-1 (ELAM-1) and intercellular adhesion molecule-1 (ICAM-1), which are ligands for adhesion molecules on leukocytes (integrins), and thus influence gingival leukocyte infiltration.

α2-macroglobulin (α2-M) and α1-antitrypsin (α1-AT) together comprise 90% of the serum protease inhibitors (2) and have been identified in gingival crevicular fluid (GCF) from diseased sites at about 75% of their serum levels (3). Transferrin (TF) is a serum-derived iron-binding glycorprotein (4) which in GCF might function as an antibacterial agent by producing an iron-limiting environment (5). TF has been reported in GCF at levels similar to those of α2-M and α1-AT (3). Recent evidence suggests that, in addition to their classical roles, these proteins can modulate the immune responses via several pathways: α2-M binds important effector molecules such as cytokines and cell receptors which inhibit immunological reactions (6); α1-AT has been implicated in the inhibition of lymphocyte responses, complement activation and neutrophil migration (7); and TF is involved in T-cell transformation and macrophage activation (8).

The dynamics of these acute-phase proteins, and the cytokine IL-1 in GCF, were compared with leukocyte infiltration and expression of the endothelial cell adhesion molecules ICAM-1 and ELAM-1, during 21-day experimental gingivitis episode (9).

Material and Methods

Twelve healthy students stopped toothbrushing for 21 d. At baseline (d 0), and after 4, 7, 11, 14, 17 and 21 d of undisturbed plaque accumulation and following the reinstitution of oral hygiene procedures (d 28 and 35), clinical changes were recorded using a Plaque Index (10) and a modified Gingival Index (11), and GCF was sampled using Whatman grade-4 paper strips (2 × 13 mm) (12) for 30 seconds. Quantification of GCF volume was performed using the Periotron 6000 (Harco). The strips were eluted into 1 ml of phosphate-buffered saline and 200 μl aliquots were analyzed for the α2-M, α1-AT and TF. For the IL-1 bioassay, GCF was eluted from the paper strips by incubation for 30 minutes at 4°C in 150 μl of RPMI 1640 medium with supplements. Indirect competitive immunoassays were developed for the quantification of α2-M and TF. α1-AT was assayed using a double-antibody sandwich assay. For each individual, a mean values was obtained for each sampling incidence for α2-M, α1-AT and TF levels (in ng per 30-s sample).

IL-1 activity was determined by bioassay using

the IL-1-sensitive cell line D10(N4)M (13) except that cell proliferation was determined by a colorimetric method (14). The potency of the stimulatory activity in the eluate was determined from dose-response curves obtained with a recombinant human DNA-derived IL-1 beta (rhIL-1β) standard, using the computer program ALLFIT (15). Gingival biopsies (2 × 2 mm) were taken from the first molar buccal gingiva on d 0, 7, 14 and 21 and embedded in Tissue Tek, snap frozen in liquid nitrogen after which serial 8 μm thick sections were cut. Frozen sections were immunoperoxidase-stained using a panel of monoclonal antibodies for Langerhans cells, T-helper, T-suppressor, primed T (CD45RO), pan-T and B cells, monocyte/macrophages, neutrophils, HLA-DR, ICAM-1 and ELAM-1. Leukocytes in the sections were counted at ×400 magnification in defined areas. Langerhans cells and other infiltrating cells within the epithelium were counted and the area of sectioned oral epithelium (AOE) determined, using computer assisted image analysis. Three adjacent areas within each section were counted and the mean number of positive cells per biopsy was calculated and expressed as either cells per 0.1 mm^2 of connective tissue or of AOE. HLA-DR, ICAM-1 and ELAM-1 were scored using a grading system for specified areas of the section.

Results

Following withdrawal of oral hygiene procedures the plaque index rose rapidly and was followed by the gingival inflammatory index. GCF IL-1 peaked within 7 d of the inflammatory episode ($p < 0.05$). α2-M, α1-AT, TF and GCF volume increases matched the gingival inflammation index. α2-M levels throughout the study were markedly higher than the levels of α1-AT ($p < 0.05$).

The term sulcular epithelium (SE) was used to include both the JE and oral sulcular epithelium. Langerhans cell (LC) numbers increased from baseline and peaked at d 14 ($p = 0.03$; Mann-Whitney). HLA-DR staining increased from baseline and peaked at d 7, after which the HLA-DR$^-$ cellular infiltrate started to fall, reaching baseline levels by d 21. Pan-T cells and PMN also peaked at d 7 in the SE, then reduced as inflammation progressed. Expression of vascular ICAM-1 and ELAM-1 increased at or before d 7, which coincided with the peak in IL-1 levels and T-cell infiltration. ICAM-1 expression in junctional epithelial keratinocytes peaked at d 7 and exhibited a gradient effect in the density of ICAM-1 from the crevicular towards the basal region of the epithelium.

Discussion

During the initiation of gingival inflammation many immune and inflammatory events take place at different time points. IL-1 appears to peak at d 7, as does upregulation of ELAM-1, ICAM-1, HLA-DR and leukocyte infiltration. The changes in acute-phase proteins appear to follow changes in the inflammatory index and probably reflect the changes in vascular permeability. α2-M levels throughout the study were markedly higher than the levels of α1-AT and may reflect the fact that α2-M can be locally produced by resident cells or infiltrating macrophages (16).

Sulcular neutrophil numbers decreased as gingivitis progressed and only recovered to baseline levels at d 21, suggesting PMN movement into the gingival crevice, presumably by chemotaxis. The spatial and temporal changes in PMN numbers illustrate the difficulty in interpretation using sequential biopsies. The flux of cells into the crevice, or back into the connective tissue, or arriving from the blood vessels, cannot easily be determined without time-lapse video analysis. However, immunocytochemistry and GCF ELISA techniques are valuable tools in the investigation of immune and inflammatory processes in experimental gingivitis and may elucidate the role of specific cells and soluble factors in the initiation and pathogenesis of periodontal disease.

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The ability of gingival crevicular fluid acute phase proteins to distinguish healthy, gingivitis and periodontitis sites

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Abstract. 3 acute phase proteins, from the local gingival inflammatory response, were examined for their ability to distinguish healthy, gingivitis and periodontitis sites. Indirect competitive immunoassays were developed for the quantification of α2-macroglobulin (α2-M) and transferrin (TF), and for α1-antitrypsin (α1-AT), a double antibody sandwich assay was produced. Healthy (25), gingivitis (31) and periodontitis (28) sites were sampled with filter paper strips (2 × 13 mm) and the volume assessed with the Periotron 6000. The samples were eluted in phosphate-buffered saline and analyzed for α2-M, α1-AT and TF. The results were expressed as absolute amounts per sample (ng/30 s) and on a concentration basis (ng/μl of GCF). Higher GCF absolute amounts of α2-M, α1-AT and TF were consistently obtained from diseased (gingivitis and periodontitis) sites than healthy sites ($p < 0.005$). Absolute amounts of GCF α2-M, α1-AT and TF were increased in periodontitis sites over gingivitis sites, although these differences were not statistically significant ($p > 0.1$). When the results were expressed on a concentration basis, α2-M levels from diseased sites were significantly higher than healthy sites ($p < 0.01$). In addition, GCF TF concentration was increased in periodontitis compared to healthy sites ($p = 0.03$).

Key words: acute phase proteins; α2-macroglobulin; α1-antitrypsin; transferrin; ELISA; gingival crevicular fluid.

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Host proteases released into the gingival crevice are considered potentially harmful to the surrounding tissues (Wilton 1986). These proteolytic enzymes are mainly of lysosomal origin and are derived from phagocytic cells that migrate into the gingival crevice region during inflammation (Attström & Egelberg 1970). The activity of these enzymes is modulated by specific inhibitors either locally produced or circulating in plasma (Condacci et al. 1982, Condacci et al. 1988, Giannopoulou et al. 1990). α2-macroglobulin (α2-M) and α1-antitrypsin (α1-AT) are the main serum protease inhibitors and they account for approximately 90% of its inhibitory capacity. Together they neutralise the majority of harmful proteases from each of the four catalytic classes (Ohlsson et al. 1973, Van Leuven 1982, Sandholm 1986). α2-M is a high molecular weight plasma protein (MWt: 720 kd) and its concentration in plasma is approximately 2–4 mg/ml. α1-AT is of much lower molecular weight (MWt: 55 kd) but has a similar or higher plasma concentration to α2-M (Cimasoni 1983, Sandholm 1986). Both serum protease inhibitors have been identified in material from the gingival crevice by Schenkein & Genco (1977) who reported gingival crevicular fluid (GCF) concentrations of α2-macroglobulin and α1-antitrypsin from diseased sites at about 70% of those found in serum. Condacci et al. (1982) and most recently Sengupta et al. (1988) reported decreasing absolute values of α2-M in GCF samples with the resolution of inflammation. Skalerič et al. (1986) reported that the concentration of α2-M in GCF decreased with increasing gingival inflammation and alveolar bone loss. The α1-AT to transferrin (TF) ratio in GCF and blood samples has been compared and higher mean ratios were found in GCF, although these did not correlate with the severity of periodontal disease (Åsman et al. 1981).

TF is a serum derived iron binding glycoprotein (MWt: 81 kd) whose main function in man is the transport of iron between sites of absorption, storage, utilisation and excretion (Aisen 1980). In GCF, it might function as an antibacterial agent by producing an iron limiting environment (Curtis et al. 1989). TF has been reported in GCF at levels similar to those of α2-M and α1-AT (Schenkein & Genco 1977). Other workers found that the dilution of TF in GCF is higher than that of α1-AT (Åsman et al. 1981). α2-M in GCF differs from TF and α1-AT as it is considered to be both serum derived and locally produced by fibroblasts and macrophages (Condacci et al. 1988, Giannopoulou et al. 1990, White et al. 1980). TF and α1-AT levels are predominantly serum derived and may be a more sensitive marker of vascular leakage into the crevice. Much interest has focused upon the main functions of these proteins i.e. protease inhibition (α2-M, α1-AT) and iron binding (TF). Recent evidence however, sug-

gests that these proteins are components of the acute phase of inflammation and modulate the immune responses via several pathways. Apart from inhibiting proteolytic reactions, α2-M binds important effector molecules such as cytokines, and cell receptors which inhibit immunological reactions (James 1980, James 1990). α1-AT has been implicated in the inhibition of lymphocyte responses, complement activation and neutrophil migration (Breit & Penny 1980). TF is involved in T-cell transformation and macrophage activation (Brock & Mainou-Fowler 1983, Hamilton et al. 1984). Thus, detection of these acute phase proteins in GCF may be informative in determining host responses in periodontal disease. This study set out to determine suitable assays for the precise quantification of α2-M, α1-AT and TF in GCF samples and to test their ability to differentiate between healthy, gingivitis and periodontitis sites.

Material and Methods

Clinical criteria

The Lobene gingival index (GI) (Lobene et al. 1986) was used for the assessment of gingival inflammation. Sites with gingival scoring of 0 or 1 and pocket depth (PD) not exceeding 2 mm were categorised as 'healthy'. GI between 2 and 4, and PD less than or equal to 3 mm were the criteria for the 'gingivitis' sites, whereas sites with PD exceeding 3 mm were designated 'periodontitis' sites. The number of sites sampled for each protein is shown in the respective tables (Tables 1–3).

Gingival crevicular fluid sampling and processing

Whatman grade 4 paper strips (2 × 13 mm) were used for GCF collection (Griffiths et al. 1988). The individual crevicular site was gently air-dried and any supragingival plaque was removed. The area was carefully isolated to prevent saliva contamination of the samples. The paper strip was inserted into the crevice until mild resistance was felt, and left for 30 s. Care was taken in order to avoid mechanical injury of the tissues. Strips contaminated by blood were discarded. After GCF collection, the paper strip was transferred to the chairside-located Periotron 6000 (Harco Electronics, Winnipeg) for the quantification of fluid volume. The jaws of the periotron were wiped with pure methanol between sequential readings. The strips were then placed in individual sterile bijoux and stored at −30 C until further processing. Subsequently, the strips were eluted into 1 ml of phosphate buffered saline containing 0.1% bovine serum albumin and 0.05% Tween 20, for 1 h at room temperature using a rotating mixer. The strips were then discarded and the eluate aliquoted and stored at −70 C until used. 200 μl aliquots were analyzed subsequently for the quantification of α2-M, α1-AT or TF. Different GCF samples were used for each acute phase protein.

Calibration of the Periotron 6000

Prior to commencement of this study a calibration graph was constructed for the Periotron 6000 in order to transform the periotron digital readings for each paper strip into volumes and also to assess the accuracy of the instrument. Known volumes of serum dilution 1:1 in PBS were delivered to Whatman grade 4 paper strips with a Hamilton microsyringe in volumes ranging from 0.2 to 1 μl, in 0.2 μl increments. Each measurement was performed 6 times and the mean value for each volume was used in a linear regression analysis from which the slope and intercept were used to determine the volumes of gingival crevicular fluid collected.

α2-M, α1-AT and TF quantification

All constituents were assayed by enzyme immunoassays. 2 types of assay were used.

(a) *Indirect competitive immunoassay to quantify α2-M and TF.* The method employed was a modification of the technique described previously by Altschuh & Van Regenmortel (1982). Briefly, the antigen, human α2-M (Sigma) or TF (Sigma), was coated onto a 96-well polystyrene microtitre plate (Immulon I, Dynatech laboratories, Virginia). The experimental samples and the corresponding antibody, goat anti-α2-M (Sigma) or sheep anti-TF (Scottish Antibody Production Unit (SAPU)), were added. The principle of this assay is that the solid and the liquid phase antigen will compete for the binding of the antibody which results in the sample concentration being inversely proportional to the amount of the antibody bound onto the solid phase antigen. The sample antigen was quantified indirectly by the addition of a peroxidase conjugated anti-sheep/goat IgG (SAPU).

(b) *Sandwich immunoassay to quantify α1-AT.* A modification of the technique described by Hetherington et al. (1983) was applied. Goat anti-α1-AT (Calbiochem) was coated onto the polystyrene microplate. The experimental sample was added and any antigen present was captured by the immobilised antibody. Incubation with the second anti-α1-AT (rabbit) (Calbiochem) was followed by addition of peroxidase conjugated anti-rabbit IgG (SAPU).

In both ELISA methods, each plate included positive and negative controls and serial dilutions of purified antigen (α1-AT: Calbiochem), to permit construction of a standard curve from which sample antigen quantities could be estimated. Visualisation at 490 nm was achieved by incubation with the substrate (o-phenylenediamine) (Sigma) and stopping the reaction with H_2SO_4.

The results were expressed both as ng of the substance per 30 s sample and on a concentration basis as ng per μl of GCF.

Statistical analysis

Values for GCF levels of α2-M, α1-AT and TF were markedly skewed and required logarithmic transformation prior to statistical analysis using the *t*-test. Due to this skewed distribution, confidence intervals are shown rather than the standard error or standard deviation. Significant differences of α2-M, α1-AT and TF mean levels among the clinical groups were determined by a two-sample *t*-test on the $\log_{10}$ transformed data. Data was analyzed using the 'Minitab' statistical package on the IBM PC computer.

Results

The mean values and standard errors of the clinical indices and GCF volumes for the three clinical groups are shown in Tables 1–3. Tables 1–3 also demonstrate the means and 95% confidence intervals of the levels of α2-M, α1-AT and TF respectively, for both methods of expressing the results (ng per 30 s sample or ng/μl of GCF). Statistical analysis revealed higher absolute amounts of α2-M (ng per 30 s sample) in samples from gingivitis ($p=0.0004$) and periodontitis ($p=0.0001$) sites than in samples from healthy sites. However,

Table 1. α2-macroglobulin content is expressed as ng/μl of GCF and as ng/30 s sample. figures in parenthesis indicate the 95% confidence intervals

Parameter	Site		
	healthy ($n=25$)	gingivitis ($n=25$)	periodontitis ($n=28$)
GI	0.28±0.09	2.64±0.14	3.39±0.13
PD	1.12±0.07	2.28±0.15	4.89±0.28
GCF (μl)	0.28±0.03	0.35±0.04	0.43±0.04
α2-M (ng/μl) of GCF	420 (218–807)	1368 (746–2506)[a]	2612 (1603–4256)[a]
α2-M (ng/30 s) sample	83 (44–159)	386 (231–647)[b]	887 (516–1459)[b]

GCF volumes are expressed in μl and standard errors are given. The mean clinical indices. GI and PD are shown with standard errors. Sample size is indicated under each clinical diagnostic category in parenthesis.
[a] Significantly different from healthy sites $p<0.01$.
[b] Significantly different from healthy sites $p<0.001$.

there was no significant difference ($p=0.09$) in α2-M levels (ng per 30 s sample) from gingivitis and periodontitis sites (Table 1). Significantly higher values of both α1-AT and TF, in ng per 30 s sample, were obtained for gingivitis ($p<0.005$) and periodontitis sites ($p<0.0001$) when compared to healthy sites. Although periodontitis sites had consistently higher mean α1-AT and TF levels (ng per 30 s sample) than gingivitis sites, no significant differences ($p>0.2$) were demonstrated between the two disease groups (Table 2, 3).

When the results were expressed on a concentration basis (ng/μl of GCF), α2-M showed a statistically significant increase at diseased (gingivitis and periodontitis) over healthy sites ($p<0.01$). When GCF TF concentration of the three clinical groups were compared, the periodontitis sites showed an increase over healthy sites ($p=0.03$), but no other significant differences pertained ($p>0.1$). α1-AT concentration levels among the three clinical groups (Tables 1–3) were not significantly different ($p>0.1$).

Table 2. α1-antitrypsin content is expressed as ng/μl of GCF and as ng/30 s sample. figures in parenthesis indicate the 95% confidence intervals

Parameter	Site		
	healthy ($n=24$)	gingivitis ($n=22$)	periodontitis ($n=22$)
GI	0.17±0.08	2.64±0.12	3.14±0.17
PD	1.04±0.04	2.32±0.10	5.02±0.30
GCF (μl)	0.18±0.03	0.27±0.04	0.37±0.07
α1-AT (ng/μl) of GCF	192 (113–326)	332 (224–492)	274 (192–392)
α1-AT (ng/30 s) sample	22 (15–33)	68 (49–94)[a]	81 (49–134)[b]

GCF volumes are expressed in μl and standard errors are given. The mean clinical indices. GI and PD are shown with standard errors. Sample size is indicated under each clinical diagnositc category in parenthesis.
[a] Significantly different from healthy sites $p<0.005$.
[b] Significantly different from healthy sites $p<0.001$.

Table 3. Transferrin content is expressed as ng/μl of GCF and as ng/30 s sample. figures in parenthesis indicate the 95% confidence intervals

Parameter	Site		
	healthy ($n=29$)	gingivitis ($n=31$)	periodontitis ($n=32$)
GI	0.45±0.09	2.61±0.12	3.34±0.11
PD	1.13±0.08	2.39±0.13	5.30±0.36
GCF (μl)	0.27±0.03	0.38±0.04	0.45±0.04
TF (ng/μl) of GCF	267 (125–571)	551 (302–1005)	748 (426–1312)[c]
TF (ng/30 s) sample	41 (21–78)	149 (84–265)[a]	261 (134–508)[b]

GCF volumes are expressed in μl and standard errors are given. The mean clinical indices. GI and PD are shown with standard errors. Sample size is indicated under each clinical diagnostic category in parenthesis.
[a] Significantly different from healthy sites $p<0.005$.
[b] Significantly different from healthy sites $p<0.001$.
[c] Significantly different from healthy sites $p=0.03$.

Discussion

This study demonstrates that ELISA methodology can be used to quantify α2-M, α1-AT and TF in GCF. At healthy sites the mean concentration of α2-M, α1-AT and TF was about 10% of their expected plasma concentration (≈2–4 mg/ml) (Johansson 1979).

Our data also demonstrate that higher absolute amounts of α2-M, α1-AT and TF are to be expected in GCF from diseased sites (gingivitis and periodontitis), than from clinically healthy sites. This could be accounted for by increased crevicular fluid flow in the diseased sites as a result of increased vascular permeability, and is strongly suggested by the higher GCF volume readings obtained from diseased sites. The mean absolute amounts of α2-M, α1-AT and TF were not found to differ significantly between gingivitis and periodontitis sites. There was however a marked tendency for increased levels at periodontitis over gingivitis sites (Tables 1–3). As mean GI, PD and GCF volume were higher in the periodontitis group (Tables 1–3) the influence each factor has on the acute phase protein levels is unclear. Further investigation of the correlation of these proteins with detailed clinical indices may be profitable, especially if the GCF analysis for all the proteins could be performed on the same sample.

We could not detect the same statistically significant differences when results were expressed on a concentration basis and this is a reflection of the higher variability observed when the results are expressed in this way. α1-AT exhibited a tendency for increasing values from health to disease (gingivitis and periodontitis), although none of the differences were significant. TF however, only showed a statistically significant increased GCF concentration in periodontitis over healthy sites. Gingivitis sites cannot be readily differentiated from healthy or periodontitis sites by GCF TF concentration. In contrast, GCF α2-M concentration was significantly increased in both gingivitis and periodontitis sites when compared to healthy. There is evidence that α2-M

may be produced locally by gingival fibroblasts (Condacci et al. 1988) and possibly macrophages (White et al. 1980) and this local contribution may be significant (Giannopoulou et al. 1990).

Our results are consistent with previous investigations of α2-M. Condacci et al. (1982) and Sengupta et al. (1988), which demonstrated that the absolute amounts of α2-M were increased at sites with higher degrees of gingival inflammation. When however, Condacci et al. (1982) and in an another study Skalerič et al. (1986) expressed their results on a concentration basis (μg/mg and g/l of GCF respectively) they reported, in contrast to our results, that the specific content of the inhibitor decreased as the gingival index and alveolar bone loss increased. This apparent inconsistency could be due to the different methods of GCF collection employed. Condacci et al. (1982) collected GCF using filter paper strips for 3 to 5 min and Skalerič et al. (1986) sampled using microcapillary tubes. Both methods of GCF collection are thought to irritate the gingival tissues (Cimasoni 1983) and could thus increase vascular permeability in the area, resulting in a dilution of the static GCF. In our study we used a less invasive method of GCF collection (paper strips for 30 s) that minimises irritation and microleakage of the subepithelial microvasculature. Expression of the results as amount of GCF constituent per 30 s sample does not include the volume of GCF in its calculation and may thus be subject to less variance and is probably less sensitive to alterations caused by evaporation or contamination of the sample which would appear to be unpredictable, irrespective of the sampling technique used.

It is interesting to note that levels of α1-AT in GCF are generally much lower than levels of both α2-M and TF although its concentration in plasma is similar or higher than the concentration of these other proteins. We would have expected similar or higher amounts of α1-AT in the inflamed gingival crevice, as its lower molecular weight would facilitate its extravasation. The increased levels of α2-M may be due to local production by fibroblasts and macrophages (Condacci et al. 1988, Giannopoulou et al. 1990, White et al. 1980) and a similar process may occur for TF within the gingival tissues. α2-M, α1-AT and TF (Brock & Mainou-Fowler 1983, Breit & Penny 1980, James 1990) are now recognised as being capable of regulating immune responses and their presence in the gingival crevice may reflect their involvement in local immune and inflammatory responses.

In conclusion, we have demonstrated that precise quantification of α2-M, α1-AT and TF is possible in microlitre amounts of GCF. Although gingivitis and periodontitis sites could not be differentiated by their α2-M, α1-AT and TF levels, they both had markedly higher levels than healthy sites. Gingival inflammation per se may be a major factor governing GCF α2-M, α1-AT or TF levels rather than increased pocket depth.

Zusammenfassung

Die Möglichkeit von akute-Phase-Proteinen aus der Sulkusflüssigkeit zur Unterscheidung von gesunden, Gingivitis- und Parodontitis-Zähnen

Drei akute-Phase-Proteine, aus der lokalen gingivalen Entzündungsreaktion, wurden hinsichtlich ihrer Fähigkeit zur Unterscheidung von gesunden, Gingivitis- und Parodontitis-Zähnen untersucht. Indirekte kompetitive Immunoassays wurden für die Quantifizierung von α2-Makroglobulin (α2-M) und Transferrin (TF) und für a1-Antitrypsin (α1-AT) entwickelt. Ein doppel-Antikörper-Sandwichassay wurde hergestellt: bei gesunden (25), Gingivitis- (31) und Parodontitis- (28) Zähnen wurden mit Filterpapierstreifen (2 × 13 mm) Proben entnommen und das Volumen mit dem Periotron 6000 gemessen. Die Proben wurden in Phosphat gebufferter Kochsalzlösung eluiert und auf α2-M, α1-AT und TF hin analysiert. Die Ergebnisse wurden als absolute Menge pro Probe (ng/30 s) und auf der Basis einer Konzentration (ng/μl von GCF) ausgegeben. Höhere absolute GCF-Mengen von α2-M, α1-AT und TF wurden einheitlicher von erkrankten (Gingivitis und Parodontitis) Zahnflächen erhalten als von gesunden Zahnflächen ($p < 0.005$). Absolute GCF-Mengen von α2-M, α1-AT und TF waren bei Parodontitis Zahnflächen über Gingivitis-Zähnen erhöht, obgleich diese Differenzen nicht statistich signifikant waren ($p > 0.1$). Sobald die Ergebnisse auf der Basis einer Konzentration ausgegeben wurden, war das α2-M Niveau von erkrankten Zahnflächen signifikant höher als von gesunden Zahnflächen ($p < 0.01$). Zusätzlich war die GCF TF Konzentration bei Parodontitis, verglichen zu gesunden Zahnflächen, erhöht ($p = 0.03$).

Résumé

Aptitude des protéines de la phase aiguë dans le fluide gingival à différencier les sites en bonne santé, avec gingivite et avec parodontite

L'aptitude de 3 protéines de la phase aiguë, provenant de la réaction inflammatoire gingivale locale, à différencier les sites sains, avec gingivite et avec parodontite a été étudiée. Des dosages immunologiques indirects compétitifs ont été conçus pour doser la macroglobuline-α2 (α2-M) et la transferrine (TF); pour l'antitrypsine-α1 (α1-AT), un double dosage anticorps sandwich a été produit. Dans des sites en bonne santé (25), avec gingivite (31) et avec parodontite (28), des prelevements ont été faits au moyen de bandelettes de papier filtre (2 × 13 mm) et le volume recueilli a été mesuré avec le periotron 6000. Les échantillons ont été séparés dans une solution salée tamponnée au phosphate et analysés pour rechercher α2-M, α1-AT et TF. Les résultats ont été exprimés en quantités absolues par échantillon (ng/30 s) et en concentrations (ng/μl de fluide gingival). On a régulièrement obtenu des quantités absolues d'α2-M, α1-AT et TF plus élevées dans le fluide gingival de sites atteints (gingivite et parodontite) que dans celui des sites sains ($p < 0.005$). Les quantités absolues d'α2-M, α1-AT et TF dans le fluide gingival étaient augmentées dans les sites avec parodontite par rapport aux sites avec gingivite, mais ces différences n'étaient pas statistiquement significatives ($p > 0.1$). Quand les resultats étaient exprimés en concentrations, les niveaux d'α2-M dans les sites atteints étaient significativement plus élevés que ceux des sites sains ($p < 0.01$). Enfin, les concentrations de TF dans le fluide gingival étaient augmentées dans la parodontite par rapport aux sites sains ($p = 0.03$).

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Acute-phase proteins in gingival crevicular fluid during experimentally induced gingivitis

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Adonogianaki, E., Moughal, N.A., Mooney, J., Stirrups D. and Kinane D.F: Acute-phase proteins in gingival crevicular fluid during experimentally induced gingivitis. *J Periodont Res.*

Abstract - The dynamics of four acute-phase proteins were investigated in gingival crevicular fluid (GCF) during the course of a 21 day experimental gingivitis study. These acute-phase proteins were the protease inhibitors α2-macroglobulin (α2-M) and α1-antitrypsin (α1-AT) and the iron-binding proteins transferrin (TF) and lactoferrin (LF). 6 healthy volunteers ceased all oral hygiene procedures for 3 weeks. GCF was sampled at seven day intervals from two sites per subject by paper strips for 30 s during the experimental gingivitis period and for two additional weeks after the reinstitution of oral hygiene. The mainly serum derived α2-M, α1-AT and TF exhibited very similar dynamics which reflects their common origin in GCF. Their levels increased significantly from baseline and remained high for at least one week after the reinstitution of oral hygiene measures (repeated measures MANOVA; α2-M: $p=0.015$; α1-AT: $p=0.012$; TF: $p=0.02$). This probably reflects increased vascular permeability in the gingivae and, to a lesser degree, local production by gingival inflammatory cells. In contrast to the serum derived acute-phase proteins, the neutrophil derived LF rose significantly from baseline (repeated measures MANOVA; $p=0.001$) but dropped rapidly after the reinstitution of oral hygiene measures. This could be because dental plaque was removed and thus neutrophil chemotactic agents in the crevice were decreased.

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Running title: GCF Acute-phase proteins in gingivitis

Key words: gingivitis, gingival fluid, acute-phase proteins

Chronic inflammation is a persistent feature of periodontal diseases and it is now established that accumulation of bacterial plaque is responsible for the initiation and maintenance of the inflammatory process. Loe, Theilade and Jensen's (1) experimental gingivitis model has been applied extensively and has proven invaluable in the study of gingival inflammation.

Sampling gingival crevicular fluid (GCF) provides a non-invasive means of obtaining and quantifying site specific biochemical parameters which may provide useful information on the inflammatory state at specific sites (2). Four GCF acute-phase proteins were investigated in this study. These were the protease inhibitors α2-macroglobulin (α2-M) and α1-antitrypsin (α1-AT), and the iron-binding proteins transferrin (TF) and lactoferrin (LF).

α2-M and α1-AT are the major serum protease inhibitors (serum concentration of approximately: 2-4 mg/ml) (3) accounting for approximately 90% of serum's inhibitory capacity (4). Together they neutralise the majority of harmful proteases from each of the four catalytic classes (5) reflecting their considerable potential function in the protease rich GCF. TF and LF are iron-binding proteins with a very similar structure but of different origin. TF is mainly serum derived (serum concentration approximately 2 mg/ml) (3) and its main function in man is the transport of iron (6). LF is present in external excretions (milk, saliva, tears) (7). In addition, LF is found in abundance in PMN secondary granules (8) but not in other leucocytes (9), and only in trace amounts in serum (10). TF and LF in GCF might function as antibacterial agents by creating an iron limiting environment (2) although LF may also exert a direct bactericidal effect independent of iron deprivation (11). Finally, the roles of the four acute-phase proteins in inflammation and their function in immune reactions is also recognised (12-15).

The above acute-phase proteins have been identified in GCF (16-21) and we have recently shown that their absolute amounts increase in diseased compared to healthy sites in cross-sectional investigations (22, 23). However, with the exception of the study of Giannopoulou and coworkers (19) who assessed α1-AT and α2-M in the same GCF sample no other studies have simultaneously measured α2-M, α1-AT, TF and LF in GCF. The present study aims to establish the dynamics of these acute-phase proteins in GCF during experimentally induced gingivitis and provide a biochemical profile of GCF.

MATERIAL AND METHODS

Subjects and design

Six healthy dental students (5 males, 1 female; age range of 22-23 years) with no clinically detectable periodontal attachment loss or pocketing >3mm in any site were enrolled in the experimental gingivitis trial. Dental students were preferred because of their excellent oral hygiene and high degree of dental awareness and compliance. The experimental gingivitis model of Löe et al. (1) was adapted for use in this study. Subjects were enrolled in the trial 10 days prior to the commencement of the experimental gingivitis period (day -10) when clinical indices were recorded. Oral hygiene instruction and prophylaxis were then given. Ten days later (day 0/baseline) all oral hygiene procedures were ceased

for 3 weeks. The subjects were then followed on days 0, 7, 14 and 21 of the experimental gingivitis period and 1 and 2 weeks after the reinstitution of oral hygiene measures. Clinical indices were recorded and GCF samples were obtained from the mesial aspect of the upper first premolars which were free of any dental restorations. Clinical indices included the Modified Gingival Index (MGI) (24) and the Plaque Index (PI) (25).

Gingival crevicular fluid sampling

Sterile Whatman grade 4 (Whatman Labsales Ltd., Maidstone, Kent) paper strips (2x13mm) were cut using a steel ruler and a scalpel and a line was drawn at 5mm indicating the length of the strip to be inserted between the Periotron jaws. The individual crevicular site was gently air-dried in an apico-coronal direction and any visible supragingival plaque was removed. The area was carefully isolated with cotton wool rolls and a saliva ejector, to avoid salivary contamination of the samples. The paper strip was introduced into the crevice until mild resistance was felt, whilst care was taken to avoid mechanical injury of the tissues. The strip was left in the crevice for 30 s and then transferred, for volume determination, to the chairside located, Periotron 6000 (Harco Electronics, Winnipeg, Canada). The Periotron was calibrated using the customised Whatman 4 paper strips each day GCF samples were collected. The strip was then stored in a labelled sterile 1ml microcentrifuge tube, and placed on ice until all sampling was completed. The strips were subsequently transported to the laboratory and stored frozen at -70°C until further processing. Prior to assaying, samples were eluted into 1ml of phosphate buffered saline containing 0.1% w/v bovine serum albumin and 0.05% Tween 20 (IB= incubation buffer) for 1h at room temperature.

Quantitation of GCF constituents

α2-M, α1-AT, TF and LF in eluates of GCF were assessed using sandwich enzyme linked immunosorbent assays (ELISA). The sandwich ELISA methodology allowed the quantification of α2-M, α1-AT, TF and LF in the same GCF sample. This permitted assessment of the levels of these proteins in GCF simultaneously during the development and reduction of gingival inflammation.

The four sandwich ELISAs (α2-M, α1-AT, TF and LF) are based on the technique described by Hetherington et al. (10). In brief, the 96-well polystyrene microplate (Immulon IV, Dynatech Laboratories, Billinghurst, Sussex) was coated with the first antibody, a goat antiserum specific to the antigen to be quantified (1:6000 dilution in carbonate/bi-carbonate buffer for α2-M, TF and LF; 1:3000 for α1-AT). The eluate of the sample was then added and any antigen present was captured by the immobilized antibody. This was followed by incubation with the second specific antiserum, developed in rabbit (1:4000 dilution in incubation buffer). Finally, the horseradish peroxidase (HRP) conjugated anti-rabbit IgG (goat) was added (1:4000 dilution in incubation buffer). Visualisation was achieved by incubation with the substrate and stopping the reaction with H_2SO_4. The plate was read at 490nm, and optical densities (ODs) obtained using the Dynatech Minireader II (Dynatech Laboratories, Alexandria, VA). Plates included serial two-fold dilutions of purified antigen for the construction of a standard curve. Only the central wells were used when running standards or samples (in triplicate). The peripheral wells were used for assaying the controls. All controls were run in duplicate except for the zero-antigen control which was run in quadruplicate. The working range of the standard for each of the above assays is shown in Table 1. Prior to assaying with the sandwich ELISAs, GCF sample eluates were diluted further in IB in order to achieve an optimal final dilution for each assay. These optimal dilution ranges for each assay are shown in Table 1.

The recovery rates of the four acute-phase proteins from Whatman grade 4 paper strips are also shown in Table 1. LF demonstrated the lowest recovery rate compared to α2-M, α1-AT and TF. GCF data, as presented in this report, were corrected to 100%, using the respective recovery rate for each acute-phase protein (Table 1).

Reagents

Purified α2-M and TF were obtained from SIGMA (SIGMA Chemical Company Ltd., Poole, Dorset) whereas LF and α1-AT were purchased from Calbiochem (Novabiochem Ltd., Nottingham). Goat and rabbit anti-α2-M, and goat anti-TF were also obtained from SIGMA. Goat anti-LF as well as rabbit anti-LF and anti-TF were obtained from Nordic Immunological Laboratories (Maidenhead, Berkshire) whereas goat and rabbit anti-α1-AT were purchased from Calbiochem. The horseradish peroxidase (HRP) conjugated anti-rabbit IgG (goat) was purchased from ICN Immunobiologicals (Lisle, IL, USA). Of the above antisera the rabbit antisera to α2-M, α1-AT, the goat anti-TF and the HRP conjugates were fractionated.

Statistical analysis

GCF constituent levels or MGI, PI scores and GCF volumes from the two sites (upper right and left first premolars) were averaged within each subject. GCF data were analyzed using the repeated measures analysis of variance and the MANOVA procedure on the SPSS/PC statistical package (26). The subject was considered as the experimental unit for the analysis of the GCF acute-phase protein data. This was justified as in experimental gingivitis studies gingival conditions are standardised at all sites within each subject prior to the initiation of the experimental period. In addition, preliminary analysis of the data demonstrated that incorporating the site in the analysis did not have a significant effect. Each protein was tested separately for differences with time. In order to satisfy the distributional requirements of the GCF data for the tests, a $\log_{10}$ transformation was required ($\log_{10}(1+x)$, x=original data value). In an attempt to investigate which days demonstrated significantly elevated GCF acute-phase protein levels since baseline, individual paired t-tests were employed on the $\log_{10}$ transformed data, comparing baseline levels of each protein to each subsequent day. A Bonferroni correction was applied and the significance level for the paired t-tests was set at 0.02 ($0.05/\sqrt{5}$, where 5=number of comparisons made) (27).

A further transformation of the logarithmically transformed data to z scores was used in this study. The z scores for each protein were produced by subtracting each data value from the grand mean of the protein and dividing by its standard deviation. This resulted in a new variable for each protein which had a grand mean of 0 and standard deviation of 1. In this way differences in overall level among the four proteins were removed permitting a clearer focus on the pattern of change during the experimental gingivitis study.

RESULTS

MGI and PI scores and GCF volumes, for the 6 subjects (mean & SE), are shown in Figure 1. MGI and PI scores demonstrated the pattern typical of experimental gingivitis studies, rising when oral

hygiene procedures ceased. After the reinstitution of oral hygiene measures, PI returned rapidly to baseline levels and MGI followed remaining, however, slightly higher than baseline. GCF volume rose, similarly to the clinical indices, with the accumulation of plaque and the development of gingival inflammation. GCF volumes remained higher than baseline after the reinstitution of oral hygiene measures.

The changes in GCF acute-phase protein levels over time for the 6 subjects are depicted in Figure 2, a to d (geometric mean and 95% confidence intervals; semi-log scale). Repeated measures analysis of variance revealed that GCF acute-phase protein levels changed significantly over time during the experimental gingivitis trial (α2-M: p=0.015; α1-AT: p=0.012; TF: p=0.02; LF: p=0.001). The results of the follow up analysis comparing individual days to baseline, using paired t-tests, are also depicted in Figure 2. Figure 3 demonstrates the z scores of the GCF acute-phase protein data (mean response for the 6 subjects). Figures 2 and 3 indicate that α2-M, α1-AT and TF in GCF demonstrated a very similar pattern, rising and remaining significantly higher than baseline even after the reinstitution of oral hygiene measures. In contrast, LF in GCF dropped rapidly after the reinstitution of oral hygiene measures returning to almost baseline levels.

DISCUSSION

The clinical findings of this study are consistent with previous experimental gingivitis trials. Upon cessation of oral hygiene measures plaque accumulated, gingival inflammation developed and simultaneously GCF volume rose. Interestingly, despite the 'clinical' improvement in gingival conditions during the healing period (days 28 and 35) vascular permeability persisted as shown by only a slight drop in GCF volumes.

In this study GCF data were reported as absolute amounts in ng/30s sample. The rationale for expressing results of GCF constituents as absolute amounts (ng/30s) has been discussed extensively (28, 29). Moreover, in the present study, GCF volumes were in the ultra low range, and thus minimal errors in GCF volume assessment would have created disproportionate flaws in concentrations of constituents (29).

The mainly serum derived α2-M, α1-AT and TF demonstrated a very similar pattern during the experimental gingivitis period, reflecting their common origin in GCF (Figs. 2 and 3). The increase in their levels in the crevice could be attributed to the development of gingival inflammation and to the resulting increased vascular permeability. This is supported by the simultaneous rise in GCF volume. In addition, increased local production could also contribute. Gingival fibroblasts synthesise α2-M (30) whereas monocytes/macrophages can produce α2-M (31), α1-AT (32) and TF (33). Although we have shown that total macrophage numbers do not change within the gingivae during experimentally induced gingivitis (34) the proportion of activated macrophages increases (35), and this may enhance the local production of α2-M, α1-AT and TF. α1-AT is produced and stored within PMN primary granules (36) and inducer T-lymphocytes, which increase with the severity of gingival inflammation (37), may also contribute to the synthesis of small amounts of TF within the tissues (38).

The dynamics of α2-M, α1-AT and TF during the healing period (days 28 and 35) demonstrated a consistent pattern still, remaining significantly higher than baseline for at least one week. This could be attributed to the maintenance of increased vascular permeability as shown by the fact that GCF volumes demonstrated only a slight decrease during the recovery period. Sustained local production by resident cells of the periodontium may also be responsible for the high levels of α2-M, α1-AT and TF after the reinstitution of oral hygiene measures, as it has been shown that clinical improvement precedes the histological resolution of gingival inflammation (39). In addition, in the case of protease inhibitors, lower degradation rates by microorganisms (40) or lower clearance rates (after binding to proteases) by macrophages/monocytes within the tissues (41), could also contribute.

GCF LF demonstrated a different overall pattern (Figs. 2 and 3), rising with the development of gingival inflammation and dropping after the reinstitution of oral hygiene measures. We have shown that LF correlates with the number of crevicular PMNs (23) and it has been shown that crevicular PMN numbers increase with the development of experimental gingivitis (42, 43). As LF is contained in only trace amounts in serum (10), the increase in its levels in GCF during the development of experimental gingivitis cannot be accounted for by increased vascular permeability. Migration of PMNs in the gingival crevice and degranulation therein is probably the major factor contributing to increased GCF LF levels. When chemotactic agents (dental plaque) are removed with the reinstitution of oral hygiene, PMN emigration rates drop and consequently LF in the crevice decreases rapidly in contrast to the mainly serum derived α2-M, α1-AT and TF.

GCF acute-phase protein data as presented in Figures 2 and 3 demonstrate the average response for the 6 subjects. Although there was small intra-subject variation in our clinical and biochemical data, a wide inter-subject variation was observed which might reflect individual variation in plaque accumulation or gingival inflammation in response to plaque. Alternatively, it could reflect an inherent variation in the local production of these acute-phase proteins. As far as α2-M and α1-AT are concerned, an interesting feature of this heterogenous response was obvious during the healing period; although most subjects sustained high levels of GCF α2-M and α1-AT after the reinstitution of oral hygiene measures, one subject demonstrated a definite drop to almost baseline levels. Interestingly, this type of response resembles the data presented by Giannopoulou *et al.* (19). These investigators showed that immunoreactive GCF α2-M and α1-AT increased with the development of experimental gingivitis but, in contrast to the present study, inhibitor levels in the crevice dropped rapidly after the reinstitution of oral hygiene. Thus, there may be differences between individuals in the course and resolution of the inflammatory process and the resulting vascular permeability. This is supported by the fact that the serum derived GCF TF decreased at the same time as the inhibitors, in the subject participating in the present study. However, one could also speculate that some individuals may present with inherently higher clearance rates of protease-inhibitor complexes within the tissues during the resolution of gingival inflammation which would be reflected by a drop in crevicular α2-M and α1-AT levels. As the ELISA methodology used in this study assesses both bound and unbound inhibitor, subjects with lower protease/inhibitor complex clearance rates would maintain high GCF α2-M and α1-AT levels during the healing period. It has been suggested that assessing bound/unbound inhibitor ratio in GCF may provide useful information on the protease inhibitory status at specific sites.

However, it should be borne in mind that in the crevice where conditions for PMN proteinase release are favourable and monocytes/macrophages are scarce (44), bound inhibitors would predominate and protease/inhibitor complexes would persist for longer time periods than in the tissues where complexes are removed rapidly upon formation (41). Therefore, bound/unbound inhibitor ratio in GCF may not be reflecting the situation within the tissues. In the gingivae, the protease/inhibitor imbalance required for damage to occur, probably arises when PMNs are closely attached to their substrate (host-tissue surfaces) and release their proteinases within closed inter-phases. The close contact would inhibit the accessibility of substances of molecular weight greater than 50 kDa (45). α2-M (Mwt: 725 kDa) would thus be completely excluded from the site of activity, whereas even if small amounts of α1-AT (Mwt:55 KDa) could reach, they would be readily inactivated by oxidative mechanisms (46-48). In this context these protease inhibitors within the gingivae may act as homeostatic regulators, functioning when PMNs release their contents in open spaces, thus being essential in preventing excessive tissue damage.

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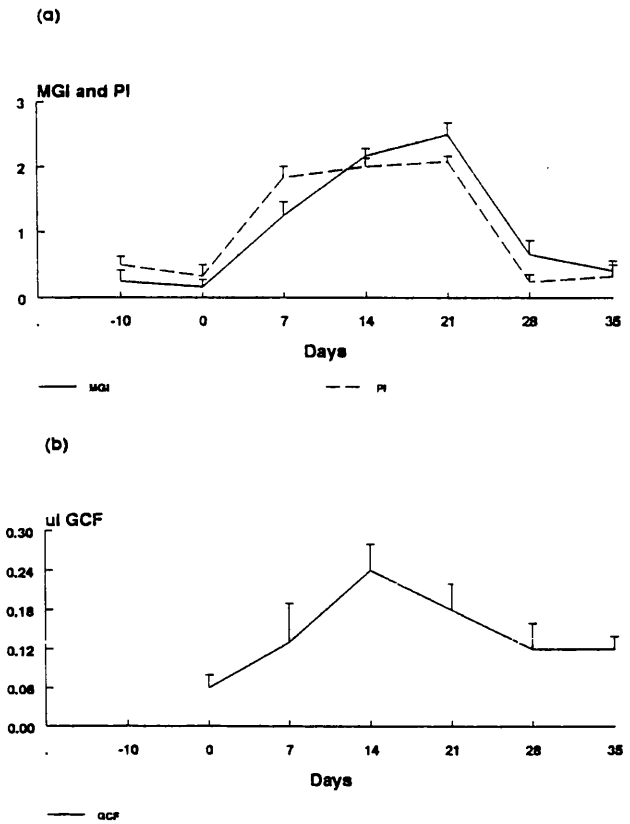

Fig. 1. (a) modified gingival index (MGI), plaque index (PI) and (b) gingival crevicular fluid (GCF) volume during the experimental gingivitis trial. The mean and standard error (n = 6 subjects) at each time point are shown.

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Legends for figures

Figure 1. (a) modified gingival index (MGI), plaque index (PI) and (b) gingival crevicular fluid (GCF) volume during the experimental gingivitis trial. The mean and standard error (n=6 subjects) at each time point are shown.

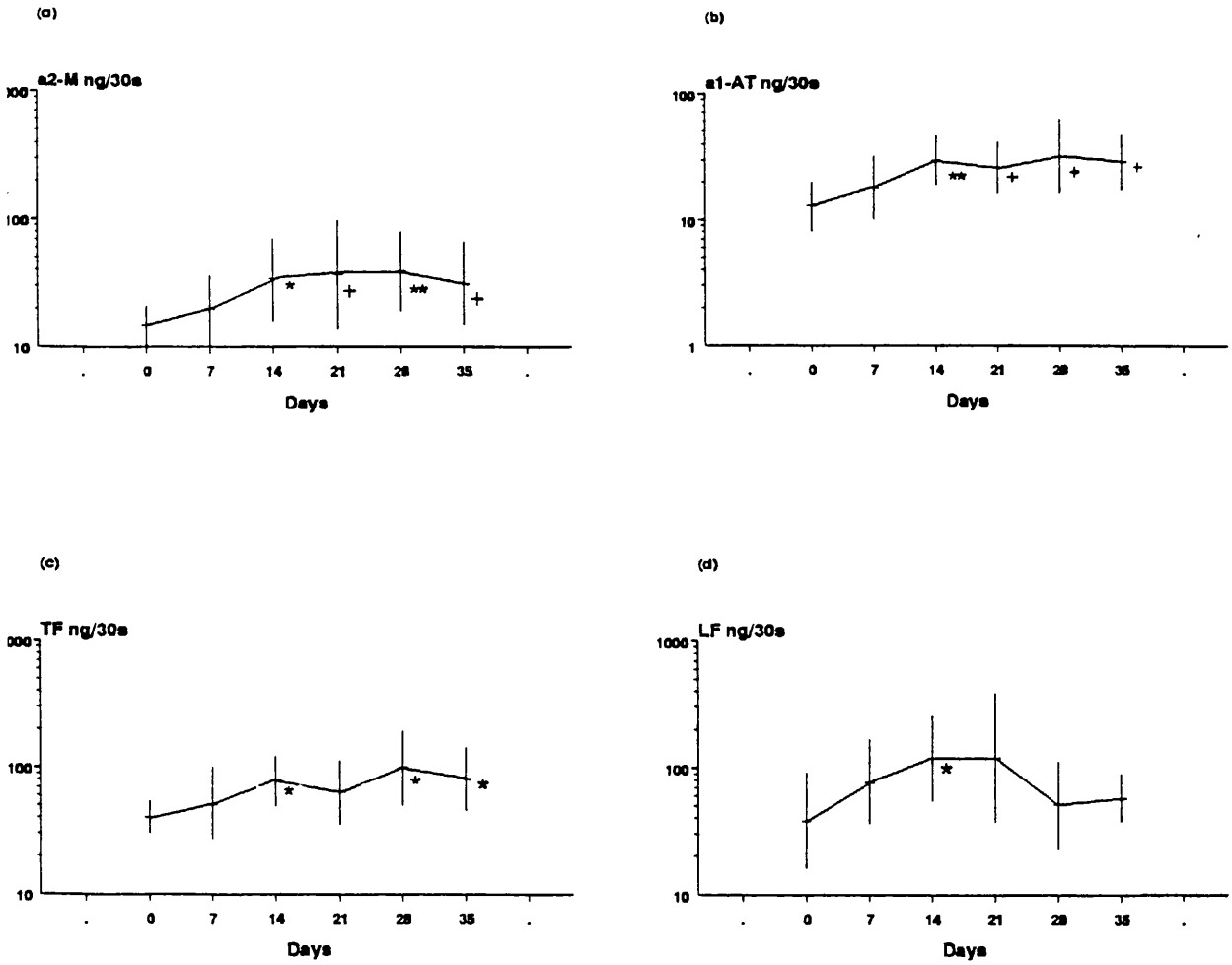
Figure 2. Acute-phase protein dynamics in gingival crevicular fluid during the experimental gingivitis trial. The geometric mean and 95% confidence intervals (n=6 subjects) at each time point are shown on a semi log scale. Data have been corrected to 100% according to the recovery rate of each acute-phase protein (Table 1). (a) α2-macroglobulin (α2-M); (b) α1-antitrypsin (α1-AT); (c) Transferrin (TF); (d) Lactoferrin (LF)
** Significantly different from baseline $p<0.01$;
* Significantly different from baseline $p<0.02$;
+ Difference from baseline failed to reach significance at $p<0.02$ but $p<0.05$.

Figure 3. Total α2-macroglobulin (α2-M), α1-antitrypsin (α1-AT), transferrin (TF) and lactoferrin (LF) changes in gingival crevicular fluid; average (n=6 subjects) z-scores of the $\log_{10}$ transformed data during the experimental gingivitis trial.

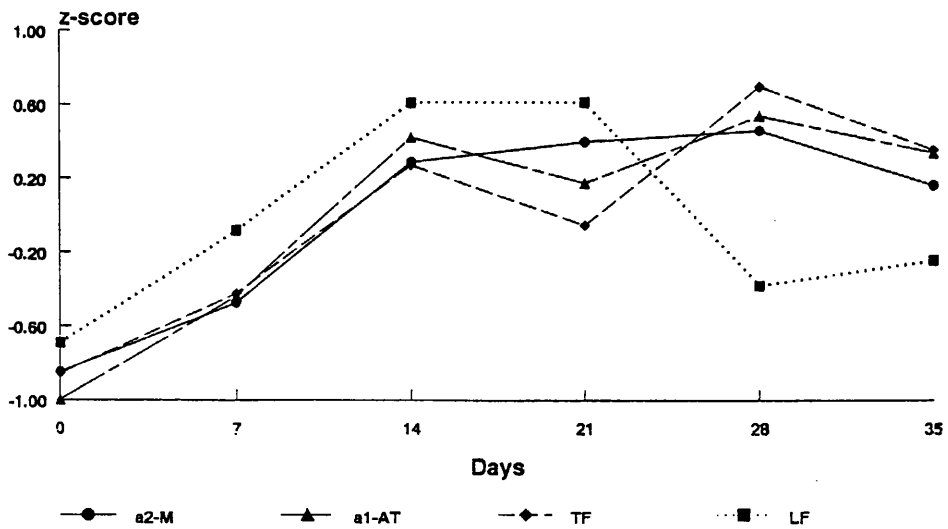
Table 1. Working range of standard antigen for the α2-macroglobulin (α2-M), α1-antitrypsin (α1-AT), transferrin (TF) and lactoferrin (LF) sandwich ELISAs.

Assay	Working range ng/ml in IB	Dilution range of GCF in IB	Recovery rate from paper strips
α2-M	100 - 1.56	1: $25x10^3$ to $5x10^4$	99.5%
α1-AT	25 - 0.78	1: $5x10^4$ to 10^5	99.4%
TF	25 - 0.78	1: $5x10^4$ to 10^5	89.9%
LF	31 - 0.49	1: 10^5 to $2x10^5$	83.4%

IB= incubation buffer



g. 2. Acute-phase protein dynamics in gingival crevicular fluid during the experimental gingivitis trial. The geometric mean and % confidence intervals (n=6 subjects) at each time point are shown on a semi log scale. Data have been corrected to 100% cording to the recovery rate of each acute-phase protein (Table 1). (a) α2-macroglobulin (α2-M); (b) α1-antitrypsin (α1-AT); (c) ansferrin (TF); (d) Lactoferrin (LF). ** Significantly different from baseline $p<0.01$; * Significantly different from baseline <0.02; + Difference from baseline failed to reach significant at $p<0.02$ but $p<0.05$.



ig. 3. Total α2-macroglobulin (α2-M), α1-antitrypsin (α1-AT), transferrin (TF) and lactoferrin (LF) changes in gingival crevicular uid: average (n=6 subjects) z-scores of the $\log_{10}$ transformed data during the experimental gingivitis trial.

Acute-phase proteins and IgG against *P.gingivalis* in peri-implant crevicular fluid: A comparison with gingival crevicular fluid.

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Abstract - The aim of the present investigation was two-fold: firstly, to determine the levels of acute-phase proteins and IgG against *Porphyromonas gingivalis* in peri-implant crevicular fluid (PICF) and their association with the clinical condition of the peri-implant mucosa; and secondly, to compare the inflammatory and immunological responses at implants and teeth as reflected by the gingival crevicular fluid (GCF) and PICF levels of acute-phase proteins and immunoglobulins. 31 partially edentulous subjects were recruited for this study. PICF was sampled from 1 healthy and 1 inflamed site from each patient; GCF was sampled from an additional 21 healthy and 27 inflamed tooth sites of the same patients. GCF and PICF were collected with paper strips (30s) and analysed using enzyme-linked immunosorbent assays for α2-macroglobulin (α2-M), α1-antitrypsin (α1-AT), transferrin (TF), lactoferrin (LF) and IgG against *P. gingivalis*. This investigation demonstrated that the absolute amounts of the acute-phase proteins and IgG against *P.gingivalis* are higher in GCF and PICF from inflamed than healthy sites. No significant differences were observed between PICF and GCF components at either healthy or inflamed sites suggesting that inflammatory events are similar in the peri-implant mucosa and gingiva and that PICF and GCF production is governed by similar mechanisms.

Running title: Acute-phase proteins and IgG in GCF and PICF

Key words: Implant, crevicular fluid, acute-phase proteins, immunoglobulins

INTRODUCTION

The long-term success of osseointegrated dental implants relies, among other things, on the maintenance of the "biologic seal" and the integrity of the peri-implant tissues (James and Lozada 1989). The peri-implant supracrestal tissues are in many ways comparable to their periodontal counterparts (Berglundh *et al.* 1991, Listgarten 1991). Like the gingival crevice at a tooth, the peri-implant crevice provides a niche for the colonisation and growth of oral microorganisms. The microflora around natural teeth and implants have been compared in partially edentulous patients, and were found to be analogous (Lekholm *et al.* 1986, Apse *et al.* 1989, Quirynen & Listgarten 1990). In addition, an animal study has shown that microbial colonization and establishment on titanium osseointegrated implants, from healthy mucosa to experimental mucositis and peri-implantitis, follow a very similar pattern to that of natural teeth (Leonhardt *et al.* 1992).

One issue which has recently attracted attention, is whether periodontal (gingiva) and peri-implant mucosa share the same inflammatory and immunological responses. The inflammatory infiltrate in peri-implant healthy and inflamed mucosa biopsies from humans has been immunohistochemically characterised and resembles that found in the gingival tissue. It comprises mainly of lymphocytes, macrophages and only very few plasma cells (Seymour *et al.* 1989). Moreover, histological studies on animals have demonstrated that the reaction of the soft tissues to early plaque formation is similar around teeth and implants (Berglundh *et al.* 1992). Long term animal studies, however, have shown that prolonged plaque accumulation and inflammation may result in a greater apical extension of the inflammatory infiltrate around implants than teeth (Ericsson *et al.* 1992) and that in experimental peri-implantitis, inflammatory cells may be found even in the adjacent bone marrow (Lindhe *et al.* 1992). Thus, a more intense inflammatory response may develop around implants when compared to teeth in response to prolonged plaque accumulation, although further human studies are needed to confirm this.

Gingival crevicular fluid (GCF) or peri-implant crevicular fluid (PICF) provides a non-invasive means of comparing the inflammatory and immunological responses around natural teeth and implants. However, in contrast to GCF which has been widely examined (Curtis *et al.* 1989), very few studies exist on PICF components and their relation to the peri-implant status (Apse *et al.* 1989, Beck *et al.* 1992).

The protease inhibitors, α2-macroglobulin (α2-M) and α1-antitrypsin (α1-AT) play an important role in the neutralisation of the proteases released in the gingival crevice (Sandholm 1986), whereas the iron-binding proteins transferrin (TF) and lactoferrin (LF) may act as antimicrobial agents in the area (Curtis *et al.* 1989). These acute-phase proteins have been previously identified in GCF (Asman *et al.* 1981, Condacci *et al.* 1982, Friedman *et al.* 1983, Skaleric *et al.* 1986, Sengupta *et al.* 1988, Giannopoulou *et al.* 1992). In addition, it has recently been demonstrated that their absolute amounts are elevated in GCF from 'diseased' compared to 'healthy' sites (Adonogianaki *et al.* 1992, Adonogianaki *et al.*

1993), and also that LF reflects polymorphonuclear leucocyte (PMN) numbers in the crevice (Adonogianaki *et al.* 1993). Thus, these acute-phase proteins may also serve as markers of peri-implant inflammatory status.

Local GCF immunoglobulin levels against putative periodontal pathogens, including *P.gingivalis*, may provide a measure of the humoral immune response at a specific site. Recently, Kinane *et al.* (1993) showed that GCF antibody titres against *P.gingivalis* were lower in deep pockets and inflamed sites than in shallow pockets or less inflamed sites. As *P. gingivalis* has been isolated also from the subgingival microflora around implants in partially edentulous patients (Lekholm *et al.* 1986, Apse *et al.* 1989, Quirynen & Listgarten 1990), PICF IgG levels against this periodontal pathogen may provide useful information on the immunological status of peri-implant sites.

The aim of this investigation was two-fold: firstly, to determine the levels of acute-phase proteins and IgG against *P.gingivalis* in PICF and their association with the clinical condition of the peri-implant mucosa; and secondly, to compare the inflammatory and immunological responses at implants and teeth as reflected by the GCF and PICF levels of acute-phase proteins and immunoglobulins.

MATERIAL AND METHODS

Subjects, sites and clinical indices

Thirty one partially edentulous patients (18 males, 13 females; age range: 23 to 84 years), who had previously been treated with osseointegrated bridges at the Brånemark Clinic (Gothenburg, Sweden) were selected to participate in this study. No subject had any history of systemic conditions which could influence the course of periodontal disease and had not been on antibiotics for the previous two months, were selected to participate in this study. The participants had at least two osseointegrated implants (*ad modum* Brånemark), which had been in function for a minimum of one year (average: 3.5 years; range: 1 to 10 years). PICF was sampled from one healthy and one inflamed non-adjacent peri-implant sites. In addition, 17 of the 31 patients contributed with one healthy and one inflamed tooth sites from which GCF was collected. Out of the remaining 14 subjects, 4 contributed with one healthy and 10 with one inflamed tooth site only. Hence, a total of 21 healthy and 27 inflamed tooth sites were sampled for intra-subject comparisons between implants and natural teeth (Table 1).

Table 1. Distribution of sites sampled in the subjects participating in this study (n=31). The resulting total number of sites in each group (healthy/inflamed, implants/teeth) is also given.

Distribution of sites

	Implant		Tooth	
Subjects	Healthy	Inflamed	Healthy	Inflamed
n=17	+	+	+	+
n=10	+	+	-	+
n=4	+	+	+	-
n=31				
	n=31	n=31	n=21	n=27

The non-invasive modified gingival index (MGI, Lobene *et al.* 1986) was used to assess inflammation and categorise the mucosa/gingiva at implants or teeth as healthy or inflamed. Sites with an MGI score of 0 or 1 were allocated to the clinically healthy group and sites with a score of 2 to 4, to the inflamed group. Presence of microbial deposits was assessed with the use of the Plaque Index system (PII, Silness & Löe 1964). Following GCF and PICF sampling, probing depth (PD) and bleeding on probing (BOP) was assessed at implants and teeth using a pressure-sensitive periodontal probe with Williams markings (0.25N; diameter: 0.45mm; Electronic Periodontal Probe, Vine Valley Research, Middlesex, NY, USA). Dichotomous scoring was used for BOP.

GCF and PICF sampling

Sterile Whatman grade 4 (Whatman Labsales Ltd., Maidstone, Kent) paper strips (2x13mm) were cut using a steel ruler and a scalpel, and a line was drawn at 5mm indicating the length of the strip to be inserted between the Periotron jaws. The individual crevicular site was gently air-dried in an apico-coronal direction and any visible supragingival plaque was removed. The area was carefully isolated with cotton wool rolls and a saliva ejector, to avoid salivary contamination of the samples. The paper strip was introduced into the crevice until mild resistance was felt or to a maximum of 1mm in deeper pockets. The strip was left *in situ* for 30s and then transferred, for volume determination, to the chairside located Periotron 6000 (Harco Electronics, Winnipeg, Canada), which was calibrated at each session using known volumes of phosphate-buffered saline in a 1:1 dilution with serum. The strip was then stored in a labelled sterile 1ml microcentrifuge tube, and placed on ice until all sampling was completed. The strips were subsequently transported to the laboratory and stored frozen at -70°C until further processing. Prior to assaying, samples were eluted into 1ml of phosphate buffered saline containing 0.05% Tween 20 for 1h at room temperature.

Quantitation of GCF and PICF constituents

α2-M, α1-AT, TF, LF, albumin (Alb) and IgG against *P. gingivalis* in GCF and PICF eluates were assessed in the same sample using enzyme linked immunosorbent assays (ELISA).

The five sandwich ELISAs for α2-M, α1-AT, TF, LF and Alb are based on the technique described by Hetherington *et al.* (1983) and modified by Adonogianaki *et al.* (1993). In brief, the 96-well polystyrene microplate (Immulon IV, Dynatech Laboratories, Billinghurst, Sussex, England) was coated with the first antibody, a goat antiserum specific to the antigen to be quantified (1:6000 dilution in carbonate/bi-carbonate buffer for α2-M, TF, LF and Alb; 1:3000 for α1-AT). The eluate of the sample was then added and any antigen present was captured by the immobilized antibody. This was followed by incubation with the second specific antiserum, developed in rabbit at 1:4000 dilution in incubation buffer, containing either 0.1% bovine serum albumin for the α2-M, α1-AT, TF and LF ELISAs (IB) or 5% proprietary milk (MARVEL) for alb (IB/M). Finally, the horseradish peroxidase (HRP) conjugated anti-rabbit IgG (goat) was added (1:4000 dilution in the respective incubation buffer, IB or IB/M). Visualisation was achieved by incubation with the substrate and stopping the reaction with H_2SO_4. The plate was read at 490nm. Plates included serial two-fold dilutions of purified antigen for the construction of a standard curve. Only the central wells were used

achieve an optimal final dilution for each assay. The optimal dilution ranges for the samples, the working range for the standard for the α2-M, α1-AT, TF and LF ELISAs as well as the recovery rate for each of the acute-phase proteins from paper strips has been given elsewhere (Adonogianaki *et al.* 1992). The working range for the albumin ELISA was 125-1.95 ng/ml and the dilution ranges for the samples are given in Table 2. Results were expressed either as ng/30s sample or ng/μg albumin.

Table 2. GCF sample dilution procedure prior to assaying by the albumin sandwich ELISAs. Samples were originally eluted in 0.5ml of IB and further diluted, as shown below in IB/M, to yield a final dilution of approximately at the range of $1/10^6$ to $1/(2x10^6)$

GCF volume (v) in μl	Assay Albumin **Dilution factor**
$v \leq 0.1$	1/50
$0.1 < v \leq 0.2$	1/100
$0.2 < v \leq 0.4$	1/200
$0.4 < v \leq 0.8$	1/400
$0.8 < v$	1/800

v= GCF or PICF volume

Specific antibody titres against *P.gingivalis* were measured by ELISA based on the method of Ebersole *et al.* (1980, 1984), using formalinised whole cells at an absorbance (OD600) which had previously been determined as optimum to coat microtitre plates. The bacterial strain used was *P. gingivalis* NCTC 11834. *P. gingivalis* was grown under anaerobic conditions (85% N_2, 10% H_2, 5% CO_2) at 37°C on Columbia blood agar. *P. gingivalis* was harvested after 5 days into phosphate-buffered saline (PBS, pH 7.4), with 1mM Na_2EDTA (PBSE), washed by centrifugation, and fixed for 1 hour in 10% formal saline. The cells were then washed twice in PBS and once in 0.1M Na carbonate-bicarbonate buffer containing 0.02% NaN_3 at pH 9.6 (coating buffer). Fixed cells were stored in coating buffer at 4°C until use. Immulon 1 plates (Dynatech) were employed because of their low protein-binding characteristics. After coating, the plates were treated with PBS containing 0.1% bovine serum albumin (BSA), 0.05% Tween 20 and 5% skimmed milk to remove background binding. Serum or GCF diluted in this buffer (excluding skimmed milk), to a concentration within the range of the calibration graph, were then added for 2 hours at 37°C, and the plates were subsequently incubated with biotin-anti-human IgG (150ng/ml) (Sigma) and thereafter with 1μg/ml extravidin-peroxidase (Sigma). The reaction was visualized using o-phenylenediamine substrate and stopped with 1M H_2SO_4. Optical densities were read at 490nm. Samples were assayed in duplicate in the central wells and peripheral wells were used as controls. Correction was made for non-specific binding and results were read from a reference line derived from serial dilutions of a reference positive control serum. Results were expressed as absolute amounts in ELISA units (Gmur *et al.* 1986) per 30s sample (EU/30s) sample or as specific amounts in EU/μg Alb.

Reagents

Purified α2-M ,TF and Alb were obtained from SIGMA (SIGMA Chemical Company Ltd., Poole, Dorset, England) whereas LF and α1-AT were purchased from Calbiochem (Novabiochem Ltd., Nottingham, England). Goat and rabbit anti-α2-M and anti-Alb, goat anti-TF, biotin-anti-human IgG and extravidin-peroxidase were also obtained from SIGMA. Goat anti-LF as well as rabbit anti-LF and anti-TF were obtained from Nordic Immunological Laboratories (Maidenhead, Berkshire, England) whereas goat and rabbit anti-α1-AT were purchased from Calbiochem. The horseradish peroxidase (HRP) conjugated anti-rabbit IgG (goat) was purchased from ICN Immunobiologicals (Lisle, IL, USA). Of the above antisera the rabbit antisera to α2-M, α1-AT, the goat anti-TF and the HRP conjugates were fractionated.

Statistical analysis

In order to control for inter-patient variability only within patient comparisons were made. GCF or PICF results were logarithmically transformed in order to satisfy their distributional requirements ($\log_{10}$ (1+x) where x= original data value). Four separate multivariate repeated measures analysis of variance (MANOVA) procedures were applied to the logarithmically transformed data to test for all proteins simultaneously for differences between: a) healthy and inflamed peri-implant mucosa (n=31 pairs), b) healthy and inflamed gingiva (n=17 pairs), c) healthy gingiva and peri-implant mucosa (n=21 pairs) and finally d) between inflamed gingiva and peri-implant mucosa (n=27 pairs). When a significant difference was detected by the analysis of variance, univariate paired t-tests were applied to the log transformed data to identify the location and direction of the statistically significant differences.

RESULTS

All of the implants that were selected for this study were well osseointegrated and none showed signs of increased rate of bone resorption.

The clinical data are depicted in Table 3. By definition, inflamed implant and tooth sites had higher MGI scores than healthy implant and tooth sites respectively. PLI was also higher at both categories of inflamed sites when compared to their healthy counterparts. Average probing depths were deeper at implants when compared to teeth, particularly at healthy sites, and in addition, average crevicular fluid volumes were higher. 67% of the healthy implant sites bled on probing compared to 14% of healthy tooth sites. The percentage of bleeding implant and tooth sites, allocated to the inflamed category was 93% and 85% respectively.

Multivariate repeated measures analysis of variance (MANOVA) demonstrated a significant effect when healthy and inflamed tooth sites ($p=0.021$) were compared for their GCF absolute amounts of α2-M, α1-AT, TF, LF, Alb (ng/30s sample) and IgG against *P.gingivalis* (EU/30s). Follow-up analysis, using univariate paired t-tests, demonstrated significantly higher levels for all six proteins (α2-M, α1-AT, TF, Alb: $p<0.001$; IgG: $p=0.036$; LF: $p=0.021$) in

GCF from inflamed compared to healthy tooth sites (Table 4). Similarly, repeated measures MANOVA demonstrated a significant effect when the absolute amounts of the six proteins in PICF from healthy and inflamed sites were compared (p=0.004). Univariate paired t-tests comparing the levels of these proteins between healthy and inflamed implant sites demonstrated that the levels of α2-M, α1-AT, TF, Alb (ng/30s) and IgG (EU/30s) were significantly higher (p<0.01) in PICF from inflamed sites (Table 4). However, although LF levels showed a tendency towards increased levels in PICF from inflamed sites this increase just failed to reach statistical significance (p=0.097) (Table 4). When the absolute amounts of each protein in fluid from healthy implant and tooth sites were compared, no significant difference were noted (MANOVA, p=0.299). Similarly, when absolute amounts from inflamed tooth and implant sites were compared, the MANOVA did not demonstrate a significant effect (p=0.06) (Table 4).

Table 3. Clinical indices at teeth and implants.

	Implant sites		Tooth sites	
	Healthy n=31	Inflamed n=31	Healthy n=21	Inflamed n=27
Score	n (%)	n (%)	n (%)	n (%)
0	25 (81)	-	17 (81)	-
1	6 (19)	-	4 (19)	-
2	-	18 (58)	-	10 (37)
3	-	12 (39)	-	16 (59)
4	-	1 (3)	-	1 (4)
	mean (SD)	mean (SD)	mean (SD)	mean (SD)
	0.193 (0.4)	2.452 (0.6)	0.190 (0.4)	2.667 (0.6)
Score	n (%)	n (%)	n (%)	n (%)
0	20 (65)	8 (26)	14 (67)	5 (19)
1	10 (32)	4 (13)	6 (29)	9 (33)
2	1 (3)	19 (61)	1 (4)	11 (41)
3	-	-	-	2 (7)
	mean (SD)	mean (SD)	mean (SD)	mean (SD)
	0.387 (0.6)	1.355 (0.9)	0.381 (0.6)	1.385 (0.9)
mm	n (%)	n*(%)	n (%)	n (%)
1	-	-	4 (19)	-
2	3 (10)	5 (17)	13 (62)	9 (33.3)
3	21 (68)	13 (43)	4 (19)	9 (33.3)
4	5 (16)	11 (37)	-	9 (33.3)
5	2 (6)	1 (3)	-	-
	mean (SD)	mean (SD)	mean (SD)	mean (SD)
	3.193 (0.7)	3.27 (0.8)	2.00 (0.6)	3 (0.8)
	n (%)	n (%)	n (%)	n (%)
	21 (67)	29 (94)	3 (14)	23 (85)
	mean (SD)	mean (SD)	mean (SD)	mean (SD)
	0.156 (0.1)	0.335 (0.3)	0.111 (0.1)	0.276 (0.2)

*1 PD value missing
Modified gingival index; PlI=plaque index; PD=probing depth; Bleeding on probing; GCF (PICF)=crevicular fluid.

Table 4. Absolute amounts of α2-macroglobulin (α2-M), α1-antitrypsin (α1-AT), transferrin (TF), lactoferrin (LF), Albumin (Alb) (ng/30s) and IgG against *P.gingivalis* (EU/30s) in crevicular fluid from healthy and inflamed tooth and implant sites. Geometric means (95% confidence intervals) are shown.

	Implant sites		Tooth sites	
	Healthy n=31	Inflamed n=31	Healthy n=21	Inflamed n=27
α2-M	19 (12-30)	49 (28-83)	13 (9-18)	50 (34-73)
α1-AT	36 (25-53)	108 (77-151)	38 (28-50)	136 (99-187)
TF	37 (25-54)	89 (64-125)	41 (29-57)	97 (69-137)
LF	19 (11-35)	32 (19-54)	32 (19-55)	60 (31-116)
IgG	90 (72-104)	125 (101-155)	82 (74-91)	136 (105-175)
Alb	1086 (661-1786)	2360 (1549-4467)	711 (436-1161)	2685 (1660-4355)

MANOVA healthy v inflamed teeth, n=17 pairs, p=0.021; all univariate comparisons significant p<0.05.
MANOVA healthy v inflamed implants, n=31 pairs, p=0.004; all univariate comparisons significant p<0.05 except for LF p=0.097.
MANOVA healthy implants v teeth, n=21 pairs, p=0.299.
MANOVA inflamed implants v teeth, n=27 pairs, p=0.06.
v=versus

When results were expressed as ng/μg Alb (α2-M, α1-AT, TF and LF) or EU/μg Alb (IgG) the picture was divergent (Table 5). Repeated measures MANOVA demonstrated a significant effect when inflamed and healthy tooth sites (p=0.011) were compared. However, univariate analysis demonstrated that the only significant differences were the reduction in IgG and TF levels in GCF from diseased sites (p=0.001 and p=0.024 respectively, Table 5). GCF α2-M, α1-AT and LF levels did not demonstrate a significant difference between healthy and inflamed tooth sites when expressed as ng/μg Alb. Similarly, MANOVA demonstrated a significant effect when PICF levels of these proteins from healthy and diseased implant sites were compared (p=0.007), but univariate analysis demonstrated a significant difference only in the reduction of the levels of IgG in PICF from inflamed compared to healthy implant sites (p=0.03). Finally, MANOVA did not demonstrate a significant effect when healthy or inflamed tooth and implant sites were compared (p=0.193 and p=0.137 respectively).

Table 5. Specific amounts of α2-macroglobulin (α2-M), α1-antitrypsin (α1-AT), transferrin (TF), lactoferrin (LF) (ng/μg Alb) and IgG against *P.gingivalis* (EU/μg Alb) in crevicular fluid from healthy and inflamed tooth and implant sites. Geometric means (95% confidence intervals) are shown.

	Implant sites		Tooth sites	
	Healthy n=31	Inflamed n=31	Healthy n=21	Inflamed n=27
α2-M	17 (10-28)	20 (12-32)	17 (10-30)	19 (11-30)
α1-AT	33 (25-45)	42 (30-60)	52 (35-76)	51 (36-72)
TF	34 (25-46)	35 (24-51)	56 (39-79)	36 (27-48)
LF	17 (10-30)	14 (8-23)	44 (22-87)	23 (12-41)
IgG	82 (53-126)	47 (31-71)	115 (72-182)	50 (32-78)

MANOVA healthy v inflamed teeth, n=17 pairs, p=0.011; significant univariate comparisons TF:p<0.05 and IgG:p<0.01.
MANOVA healthy v inflamed implants, n=31 pairs, p=0.007; significant univariate comparisons IgG: p<0.05.
MANOVA healthy implants v teeth, n=21 pairs, p=0.193.
MANOVA inflamed implants v teeth, n=27 pairs, p=0.137.
v=versus

DISCUSSION

The clinical data displayed some interesting features. Very few inflamed implant and tooth sites had an MGI score of 4, demonstrating that implants and teeth were relatively well maintained. As expected, inflamed implant and tooth sites demonstrated higher amounts of plaque than healthy sites depicting, within the limits of this cross-sectional investigation, the well-known association of plaque accumulation with inflammation of the gingival tissues but also of the peri-implant tissues. When the clinically healthy sites were probed after all sampling was completed, 3 tooth sites (14%) compared to 21 implant sites (67%) bled following the probing. This difference in bleeding frequency is most likely due to the reduced resistance to probing offered by the peri-implant mucosa in comparison to that offered by the gingiva, as a consequence of differences in terms of tissue composition, organisation and attachment to the root/implant surface (Berglundh *et al.* 1991) as well as the shape of the anchorage unit. In fact, in a recent beagle dog study (Ericsson & Lindhe 1993), in which probing with standardised pressure (0.5N; probe diameter 0.5mm) at implants and teeth with non-inflamed mucosa was histologically evaluated, it was reported that the probe penetrated on the average 1.3mm (SD 0.3) into the supracrestal connective tissue at implants, while at teeth the tip of the probe was consistently located within the zone of the junctional epithelium. The high frequency of bleeding found in the present study following probing of the 'clinically healthy' implant sites, despite the use of a probing pressure of only 0.25N, indicates that a pressure less than 0.25N may be needed in order not to traverse the apical termination of the peri-implant junctional epithelium, and hence caution must be exercised when comparing the probing data for teeth and implants in the present investigation.

The assessment of the four acute-phase proteins, Alb and also IgG against *P.gingivalis* provides a profile for GCF and PICF components of different sources and with different functions. Such a profile, especially when components are assessed in GCF or PICF from the same site, gives a picture of the inflammatory and immunological responses at a specific site. None of the constituents reported in this study have been previously determined in PICF. Similarly to GCF, PICF demonstrated protease-inhibitory capacity due to the presence of the potent protease-inhibitors α2-M and α1-AT, as well as general antimicrobial properties via the iron-binding proteins TF and LF. The presence in PICF of specific immunoglobulin against *P.gingivalis*, a putative periodontopathogen that has been isolated from peri-implant sulci (Lekholm *et al.* 1986, Apse *et al.* 1989, Quirynen & Listgarten 1990), could be due to localised induction of plasma cells of the peri-implant mucosa to IgG production and/or leakage from serum. Specific immunoglobulin in the peri-implant crevice could be involved in the opsonisation of micro-organisms and also complement activation rendering further specific antimicrobial properties to PICF.

In this investigation we decided to express results of GCF and PICF constituents in two ways, as ng/30s sample and as ng/μg Alb. The rationale for expressing results as ng/30s sample has been discussed previously (Lamster *et al.* 1986). Reporting results per unit Alb provides information on constituent levels relative to the serum derived component of GCF or PICF and may thus supply an indication of proteins derived through extravasation.

It has been shown in previous investigations that the levels of the acute-phase proteins are higher in GCF from inflamed tooth sites compared to healthy sites (Condacci *et al.* 1982, Giannopoulou *et al.* 1990, Adonogianaki *et al.* 1992). Thus, when the results were expressed as ng/30s, the increase at inflamed sites was anticipated, for the protease inhibitors α2-M and α1-AT and the iron-binding proteins TF and LF (Table 4). For α2-M, α1-AT and TF, which have high serum concentrations (2mg/ml) (Johansson 1979), increased vascularity and vascular permeability is probably the major factor contributing to higher GCF or PICF levels at inflamed sites. This is supported by the simultaneous rise in GCF or PICF volume as well as Alb at inflamed sites around both implants and natural teeth. Further support for the predominantly serum origin of α2-M and α1-AT is provided by the inability to demonstrate a significant difference between healthy and inflamed sites for both implants and teeth when the results were expressed relative to Alb (Table 5).

When results were expressed as ng/30s LF demonstrated a statistically significant increase in GCF from inflamed compared to healthy tooth sites. As LF may be considered a crevicular PMN marker (Adonogianaki *et al.* 1993) its increase in GCF at inflamed sites may be accounted for by increased PMN emigration. Although a significant difference was not shown when inflamed implant and tooth sites were compared, there was a tendency for inflamed implant sites to have lower levels of LF than inflamed tooth sites. This tendency is in line with findings reported from a recent animal study showing that lower PMN numbers may be found in inflamed peri-implant tissues compared to inflamed tooth sites (Ericsson *et al.* 1992).

The finding that inflamed tooth sites demonstrated higher antibody titres (EU/30s) of IgG against *P.gingivalis* than healthy tooth sites appears to contradict the findings of our previous study (Kinane *et al.* 1992). However, one has to consider that the present study refers to non-periodontally involved sites from well-maintained patients, whereas the previous study was on a periodontitis affected population. The increase in the absolute amounts of IgG against *P.gingivalis* may be due to increased exudation from serum by similar mechanisms as for the acute-phase proteins.

An interesting observation was the drop in TF and IgG levels and the tendency for lower LF levels at inflamed tooth sites compared to healthy sites when results were expressed as ng/μg Alb (Table 5). The drop in specific IgG levels in inflamed tooth sites may be related to the opsonisation and/or degradation by *P.gingivalis* resulting in consumption of the immunoglobulin (Kilian 1981). Furthermore, both TF and LF may bind to oral isolates, which may contribute to decreased levels of the iron-binding proteins at diseased sites (Beighton *et al.* 1992; Ellison and Geihl 1991). These events would result in a relative dilution of TF, LF and IgG compared to Alb. At diseased implant sites, however, only IgG showed a trend for lower levels when the results were expressed as ng/μg Alb, which may be related to the tendency for higher Alb levels (ng/30s) at healthy implants sites compared to healthy tooth sites. The tendency for higher albumin levels may suggest that structural and anatomical differences in implant sites result in greater leakage in healthy implant compared to healthy tooth sites. However, the fact that overall no significant differences were observed between implants and teeth (ng/30s or ng/μg Alb; Tables 4 & 5) suggests that production of PICF is probably governed by similar mechanisms to that of GCF and that inflammatory events are similar in the peri-implant mucosa and the gingiva.

In conclusion, this investigation suggests that great similarities exist in the profile of GCF and PICF constituents and analogous mechanisms seem to control inflammatory and immunological responses around both implants and natural teeth.

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Humoral immune responses to *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* in adult periodontitis and rapidly progressive periodontitis.

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Running title: Antibody avidity in periodontal diseases

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Abbreviations: AP - adult periodontitis; RPP - rapidly progressive periodontitis; Ig - Immunoglobulin; ELISA - Enzyme linked immunosorbent assay; EDTA - ethylene diamine tetracetic acid; PBS - phosphate buffered saline; BSA - bovine serum albumin.

Abstract

The relationships between various forms of periodontal disease and the avidities of serum antibodies of all three immunoglobulin classes (IgG, IgM and IgA) to *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* were investigated. Twenty-four patients with untreated adult periodontitis (AP) and twelve untreated patients diagnosed as suffering from the early-onset form of periodontitis, rapidly progressive periodontitis (RPP) were studied. The latter group were age and sex matched to healthy controls. Measurement of antibody titre was made and avidity (expressed as molarity (M)) was further assayed using the thiocyanate elution method. Avidity has previously been shown to relate to the biological function of antibody. IgM avidities to *P. gingivalis* were lower in the RPP group than in the AP group (0.54M compared to 0.74M, $p<0.001$). IgG avidities tended to be lower in the former than in the latter group (0.58M compared to 0.92M, $p=0.065$). In accordance with other workers, seropositivity was defined as an immunoglobulin titre more than twice the median level of control sera. Only two of the RPP group were seropositive. Interestingly, the seronegative RPP patients were significantly different (0.53M compared to 0.92M, $p<0.01$).

The data presented here that patients with various forms of periodontal disease appear to produce antibodies of differing avidity to *P. gingivalis* suggest that the quality of the humoral immune response to suspected periodontopathogens may have a bearing on the aetiology of periodontal disease.

Introduction

The Gram-negative obligate anaerobe, *Porphyromonas gingivalis* and the facultative anaerobe, *Actinobacillus actinomycetemcomitans*, are both considered important periodontopathogens within various forms of periodontal disease (32,37). A number of studies have demonstrated that increased serum antibody titres to one or more suspected periodontopathogens occur in periodontitis (18,27,36). However, others have reported titres that are similar to or lower than those found in control subjects (9,15,19). We have shown previously that local antibody levels are lower in deeper pockets and in more inflamed sites than in shallower pockets and less inflamed sites in adult periodontitis patients (23).

In this study we aimed to ascertain whether or not adult periodontitis (AP) could be distinguished from the early-onset form of periodontitis, rapidly progressive periodontitis (RPP) (3,28), in terms of patients' humoral immune response to these organisms. Wilton *et al* concluded in their review that the data currently available on antibody titre responses to suspected periodontopathogens, e.g. *P. gingivalis* and *A. actinomycetemcomitans* do not allow these responses to be diagnostic (35). But they suggested that the investigation of antibody avidity may be more fruitful. Thus antibody avidity and titre were examined for their potential to discriminate between these patient groups.

Antibody avidity, i.e. the net binding strength between antibodies and antigens, has been shown to be a useful indicator of the biological function of antibodies in acute and chronic infections (21), autoimmunity (29), B-cell activation (33) and vaccine development (4). Recently, it has been demonstrated that the effective binding of IgG to virulent *P. gingivalis* has a crucial role in the opsonization and phagocytosis of this organism and also in complement activation (6,7). In addition,

Lopatin et al showed that the avidity of IgG-class antibodies to *P. gingivalis* was significantly increased in adult periodontitis patients compared with healthy controls (24). No such elevation was detected in IgM-class antibodies. The authors suggested that, since human antibodies appear to be of generally low avidity compared with those in rabbits immunized with this organism, the presence of low avidity antibodies may contribute to the pathology associated with periodontal disease.

Chen et al demonstrated that IgG avidities to *P. gingivalis* were lower in rapidly progressive periodontitis patients than in control subjects. However, after treatment the avidities increased significantly to levels higher than in controls (5). They concluded that many rapidly progressive periodontitis patients do not produce protective levels of biologically functional antibody as a result of natural infection, but treatment may induce the production of such antibodies.

Whitney et al, in a recent study of the humoral immune response in rapidly progressive periodontitis, showed that these patients have lower IgG avidities to *P. gingivalis* (34). Our group has shown significantly higher IgG avidities to *P. gingivalis* in adult periodontitis patients than in seronegative control subjects (26).

These findings have prompted us to compare directly adult periodontitis and rapidly progressive periodontitis patients as well as healthy controls in order to establish whether there are differences in aetiology in terms of humoral immune response to periodontopathogens.

Materials and Methods

Bacteria

P. gingivalis NCTC 11834 was grown under anaerobic conditions

(85% N_2, 10% H_2, 5% CO_2) and *A. actinomycetemcomitans* in CO_2 at 37C on Columbia blood agar (Life Technologies, P.O. Box 35, Trident House, Renfrew Road, Paisley, PA3 4EF, Scotland, UK). *P. gingivalis* was harvested after 5 days and *A. actinomycetemcomitans* after 24 hours into phosphate-buffered saline, 1mM Na_2 EDTA, pH 7.4 (PBSE), washed by centrifugation, and fixed for 1 hour in 10% formal saline. The cells were then washed twice in PBSE and once in 0.1M Na carbonate-bicarbonate buffer containing 0.02% NaN_3 at pH 9.6 (coating buffer). Fixed cells were stored in coating buffer at 4°C until use.

Subjects

Serum was collected from twenty-four adult periodontitis patients (AP), twelve rapidly progressive periodontitis patients (RPP) and twelve healthy control subjects who were age and sex matched to the rapidly progressive periodontitis patients. The mean age of the adult periodontitis patients was 40.3 years (25-53) and this group comprised 13 males and 11 females. The mean age of the rapidly progressive periodontitis patients was 32.8 years (20-40) and this group comprised 6 males and 6 females. Samples were collected from all patients before the commencement of therapy.

ELISA

Specific antibody titres were measured by ELISA based on the method of Ebersole *et al* (10,11), using formalinized whole cells at an absorbance which had previously been determined as optimum to coat microtitre plates. Immulon 1 plates (Dynatech Laboratories Ltd., Daux Road, Billingshurst, Sussex, RH14 9SJ, England, UK) were employed because of their low protein-binding characteristics. After coating, the plates were treated with PBS containing 0.1% bovine serum albumin (BSA), 0.05% Tween 20 and 5% skimmed milk to remove background binding. Serum serially diluted in a similar buffer but without the 5% skimmed milk, was

then added and the plates were subsequently incubated with biotin-conjugated anti-human IgG, IgA or IgM (Sigma Chemical Company Ltd., Fancy Road, Poole, Dorset, BH17 7NH, England, UK) and thereafter with Extravidin-peroxidase (Sigma). Reaction was visualized using o-phenylenediamine dihydrochloride substrate and stopped with 1M H_2SO_4. Samples were assayed in duplicate and results were calculated using a regression line and derived equation from serial dilutions of a reference serum. Results were expressed as ELISA units (EU) (17).

Dissociation Assay

The dissociation assay to determine antibody avidity was performed by incubating with serum as described above, and then the wells were treated with increasing concentrations of ammonium thiocyanate (0.2-8.0M). The concentration of thiocyanate required to dissociate 50% of bound antibody was determined by linear regression analysis. This was termed the ID_{50} and provides a measure of relative avidity as previously reported (25,30).

Statistical Analysis

Mann-Whitney U-tests were used in group comparisons of avidity where medians were employed and also in group comparisons of the non-normally distributed antibody titres. Student's paired t-tests were used to assess whether differences were significant between rapidly progressive periodontitis patient data and that of age/sex matched controls. Regression analysis was used to correlate avidities and titres of antibodies to *P. gingivalis* and *A. actinomycetemcomitans* in adult periodontitis, rapidly progressive periodontitis and control groups.

Results

The median relative avidity, expressed as an ID_{50} or avidity index value equivalent to the molarity (M) of ammonium thiocyanate required to dissociate 50% of bound antibody, and median titre, expressed as ELISA units (EU), of antibody to both organisms for adult periodontitis and rapidly progressive periodontitis groups are given in Table 1. Statistically significant differences between groups were assessed using the Mann-Whitney U-test and p-values are given. The avidity of IgG tended to be higher and IgM avidity to *P. gingivalis* was significantly higher in adult periodontitis compared to rapidly progressive periodontitis patients, and also IgA titre was significantly lower in rapidly progressive periodontitis compared to adult periodontitis patients. In contrast, IgA avidity to *A. actinomycetemcomitans* tended to be higher in rapidly progressive periodontitis than in adult periodontitis and IgG and IgA titres were significantly higher in rapidly progressive periodontitis than in adult periodontitis. Similarly, the median avidity of antibody to both organisms for adult periodontitis and seronegative rapidly progressive periodontitis groups are given in Table 2, with p values for the comparisons between the two patient groups. Seronegativity here is as defined by Chen et al (5) (i.e. median titre < 2 X control median), and it should be noted that the seronegative groups for *P. gingivalis* and *A. actinomycetemcomitans* are not identical, i.e. two patients were seropositive for *P. gingivalis* and a different two patients for *A. actinomycetemcomitans*.

A comparison between seronegative rapidly progressive periodontitis patients and their age/sex matched controls in terms of IgG avidity to *P. gingivalis* is also shown in Table 2 and a paired t-test indicated that they were not significantly different.

The percentage of patients in both patient groups who were seropositive in terms of titre and avidity to *P. gingivalis* and

A. actinomycetemcomitans are shown in Table 3. The seropositivity definition is extended here to include avidity, i.e. avidity > 2x median control avidity.

A discriminant analysis of the IgG avidity for adult periodontitis and rapidly progressive periodontitis patient groups was performed (Table 4). This analysis was performed with cross-validation thereby mimicking a prospective analysis (Minitab statistical software version 8). Data are shown for the analyses with the whole rapidly progressive periodontitis group and also the seronegative rapidly progressive periodontitis group.

Correlations between titre and avidity of antibody to *P. gingivalis* and *A. actinomycetemcomitans* are given in Table 5 for adult periodontitis, rapidly progressive periodontitis and control groups. Adult periodontitis and rapidly progressive periodontitis groups show different patterns in terms of these correlations, i.e. for adult periodontitis, IgG and IgA to *P. gingivalis* are correlated and IgM to *A. actinomycetemcomitans*, whereas for rapidly progressive periodontitis, IgG and IgM to *P. gingivalis* are correlated and IgG and IgA to *A. actinomycetemcomitans*. In addition, periodontitis patients are clearly different from control subjects in these correlations.

Discussion

The present study shows that IgG and IgM antibody avidities to *P. gingivalis* were lower in rapidly progressive periodontitis patients than adult periodontitis patients, with IgA titres being significantly lower than in adult periodontitis. However, a contrasting pattern could be discerned for *A. actinomycetem-comitans*, with IgA avidity tending to be higher than in adult periodontitis and IgG and IgA titres also being significantly

higher. In addition, median avidity of IgG antibody to *P. gingivalis* tended to be lower in seronegative rapidly progressive periodontitis patients than in age/sex matched controls. As such this study supports the hypothesis that there are differences in the humoral immune response to these two organisms in rapidly progressive periodontitis and adult periodontitis, and is consistent with previous studies demonstrating that avidities to *P. gingivalis* are lower than normal in rapidly progressive periodontitis (5,34) and higher than normal in adult periodontitis (24,26) and extends our knowledge by indicating that this pattern differs for the humoral response to *A. actinomycetemcomitans*.

Rapidly progressive periodontitis has been recognized as a distinct clinical condition (3,28). In addition, an association has been demonstrated between the occurrence of rapidly progressive periodontitis and the HLA system (1,20). A correlation has been demonstrated between HLA A9 and rapidly progressive periodontitis. A number of studies have been published showing that rapidly progressive periodontitis patients differ in their humoral immune response expressed as antibody titre to suspected periodontopathogens (2,16,18,36).

A recent study by Ebersole and Kornman (14) demonstrated that *P. gingivalis* emerges as an organism in the subgingival plaque during the conversion from gingivitis to progressing periodontitis in a non-human primate model, and that this elicits a systemic antibody response specific for this microorganism. Similarly, a study by Dahlen and Slots (8) in rabbits showed that animals co-inoculated with *P. gingivalis* and *A. actinomycetemcomitans* showed significantly more severe disease than animals which were monoinfected. They conclude that the immune system acting through systemic antibodies and/or cellular mechanisms may modulate the pathogenic potential of infecting periodontal pathogens. It may be that antibody avidity has a

crucial role in this modulation.

In the light of these findings and evidence of the importance of avidity as an aspect of the biological function of antibody (6,7,31), it may be that rapidly progressive periodontitis patients constitute a sub-group which is predisposed to early and rapidly progressive disease as a result of a mechanism blocking the production and/or deployment of strongly-binding antibody to suspected periodontopathogens.

To put the differences in relative avidity of antibody between different patient groups into context in terms of relative binding strength, the scale of relative antibody responses previously employed by Ebersole et al (13) may be used. The baseline avidity index to tetanus toxoid in that study of non-human primates was 0.9M, increasing to 1.72M (a 7-fold increase in binding strength) following primary immunization, and 2.56M (a 45-fold increase in binding strength) following secondary immunization. Using the same scale of relative antibody responses, the avidity index of the RPP patients in the present study indicate that the binding strength is only 30-40% of that observed in the AP patients.

It must also be borne in mind that the mean age of the adult periodontitis patients was 40.3 years (25-53), whereas that of the rapidly progressive periodontitis patients was 32.8 years (20-40). Age matching of these groups was, by definition, impossible. However, a healthy control group age and sex matched to the rapidly progressive periodontitis patients was included since it was possible that any differences found between the two patient groups might be due to age alone.

Our findings of overall low-avidity antibodies in rapidly progressive periodontitis patients, and generally in humans, are in agreement with previous reports (5,24,26,34). However, they contrast with higher avidities of antibody to *P. gingivalis* and *A. actinomycetemcomitans* found in an early study in this field

(12). It must be borne in mind, however, that these latter data were based on samples of five and four patients respectively in a mixed periodontitis group, and that these samples may have contained an unrepresentatively high proportion of seropositive adult periodontitis patients. As the present study shows, these, and a small percentage of seropositive rapidly progressive periodontitis patients can skew analyses unless measures are taken such as dichotomization into seropositive and seronegative groups or non-parametric testing based on medians rather than means.

The data presented in Table 5 on the relationship between titre and avidity are in agreement with our previous findings (26). A clear difference can be discerned between periodontitis patients and control subjects, and there is also an indication that these relationships in terms of the responses to *P. gingivalis* and *A. actinomycetemcomitans* may also yield a useful distinction between adult periodontitis and rapidly progressive periodontitis patients. It is interesting to note that all of the correlations here were positive compared with the negative correlations between titre and avidity of antibody to *Candida albicans* found in a recent study (22). Care should, however, always be exercised in the interpretation of these correlations. For example, Table 5 also shows one significant correlation for control subjects in terms of IgM against *A. actinomycetemcomitans*. However, as 3/6 correlations were statistically significant ($p<0.05$) for adult periodontitis patients and 4/6 for rapidly progressive periodontitis patients, it is highly unlikely that these patterns of response are chance findings. On the other hand, the finding of one significant correlation out of six for control subjects could have arisen by chance.

If a precipitating factor or factors is necessary to

initiate generalized, severe periodontitis at any age, and low avidity antibody is just another factor in its pathogenesis, then the current definition of rapidly progressive periodontitis based upon a cut-off age may appear even more arbitrary. This interpretation may appear particularly relevant in the light of the work of Joynson et al (21) on acute and chronic infection in relation to antibody avidity. In the present study the rapidly progressive periodontitis patients appear as a low avidity/acute infection group whereas the adult periodontitis patients appear as a higher avidity/chronic infection group.

Similarly, it may be that antibody avidity assessments could provide an indication of severity in adult periodontitis patients. Our group has previously shown a relationship between antibody avidity to *P. gingivalis* and attachment loss experience in a longitudinal study; in that IgM antibodies were of significantly higher avidity in adult periodontitis patients who did not experience further attachment loss than in those who did (26).

The data presented here that patients with various forms of periodontal disease appear to produce antibodies of differing avidity to *P. gingivalis* suggest that the quality of the humoral immune response to suspected periodontopathogens may have a bearing on the aetiology of periodontal disease.

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Table 1: Median avidities and titres of antibodies to *P. gingivalis* and *A. actinomycetemcomitans* for AP and RPP groups. Where significant, p values for Mann-Whitney U-tests are given.

	P. gingivalis						*A. actinomycetemcomitans*					
	Avidity			Titre			Avidity			Titre		
	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA
AP	0.92	0.74	1.15	653	357	80	0.65	0.61	0.80	319	545	10
(n=24)	0.07(NS)	<0.001				0.025			0.07(NS)	<0.01		0.035
RPP	0.58	0.54	1.00	524	206	11	0.74	0.61	1.08	4810	301	144
(n=12)												

Table 2: Median avidities and titres of IgG antibodies to *P. gingivalis* and *A. actinomycetemcomitans* for AP group, seronegative RPP group and controls age/sex matched to seronegative RPP group.

	P. gingivalis	*A. actinomycetemcomitans*
	Avidity (M)	
	IgG	IgG
AP (n=24)	0.92*	0.65
RPP (n=10)	0.53*	0.70
Controls (n=10)	0.76	0.67

* indicates the pair, analysed by two sample t-tests which were significantly different at p=0.004.

Table 3: Percentage of patients seropositive in terms of titre and avidity of antibody to *P. gingivalis* and *A. actinomycetemcomitans* for AP and RPP groups. Seropositivity defined as > 2x control median. for AP and RPP groups.

	P. gingivalis						***A. actinomycetemcomitans***					
	Avidity (M)			**Titre (EU)**			**Avidity**			**Titre**		
	IgG	**IgM**	**IgA**	**IgG**	**IgM**	**IgA**	**IgG**	**IgM**	**IgA**	**IgG**	**IgM**	**IgA**
AP (n=24)	**17**	**0**	**12**	**42**	**25**	**67**	**0**	**12**	**0**	**17**	**67**	**42**
RPP (n=12)	**17**	**0**	**8**	**25**	**25**	**33**	**17**	**25**	**17**	**50**	**58**	**83**

Table 4: Discriminant analysis of AP and RPP groups with cross-validation for entire RPP group and seronegative RPP group as classified on the basis of IgG avidity to *P. gingivalis*.

		True group	
		AP (n=24)	**RPP (n=12)**
Assigned group	**AP**	**11**	**3**
	RPP	**13**	**9**
	% correct	**46**	**75**
		AP (n=24)	**sero-negative RPP (n=10)**
	AP	**14**	**1**
	sero-negative RPP	**10**	**9**
	% correct	**58**	**90**

Table 5: Correlations between titres and avidities of IgG, IgM and IgA antibodies to *P. gingivalis* and *A. actinomycetemcomitans* for AP, RPP and Control groups.

	P. gingivalis			***A. actinomycetemcomitans***		
	IgG	**IgM**	**IgA**	**IgG**	**IgM**	**IgA**
AP	**19.2%, 0.03***	**1.2% 0.61**	**36.4%, 0.002***	**6.8% 0.22**	**73.8%, 0.003***	**0.1% 0.90**
RPP	**81.6%, <0.001***	**36.1%, 0.04***	**3.1% 0.58**	**80.5%, <0.001***	**1.4% 0.72**	**36.4%, 0.04***
Control	**1.5% 0.70**	**24.0% 0.11**	**9.1% 0.34**	**14.4% 0.22**	**61.2%, 0.003***	**9.6% 0.33**

Results given as R^2 (%), p-value. Asterisks indicate statistical significance at $p<0.05$.